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THE

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ON THE GENUS ANCISTRUMA STRAND (ANCISTRUM MAUPAS)

I. The Structure and Division of A. Mytili Quenn. and A. Isseli Kahl

GEORGE W. KIDDER

College of the City of New York

Introduction

In 1867 Quennerstedt described a small ciliate from the mantle cavity of *Mytilus* under the name of *Opalina mytili*. Kent (1882) placed this form in the genus *Anoplophrya* and retained the species name given by Quennerstedt. Maupas (1883) redescribed this ciliate, calling attention to the presence of a mouth, which had been overlooked by Quennerstedt and Kent. He created the genus *Ancistrum* for the type species of Quennerstedt and described a second species, *A. veneris gallinæ*. Strand (1926), in reviewing the literature, finds the name *Ancistrum* preoccupied and therefore invalid. He proposes the name *Ancistruma* to replace the commonly employed one of *Ancistrum*.

It is the purpose of this paper to report some observations on two species of the genus *Ancistruma*, namely, *A. mytili* Quennerstedt and *A. isseli* Kahl.

In his excellent monograph on the Ancistridæ of the Gulf of Naples, Issel (1903) describes in detail an *Ancistrum* from the solitary mussel *Modiola barbata*. Although his findings were not entirely in accord with previous descriptions he believed this form to be identical to *A. mytili* and placed it in that species. Kahl (1931), however, called this form *Ancistruma isseli*. I am entirely in agreement with Kahl on this point, as will be brought out in the following pages.

This investigation was carried on at the Marine Biological Laboratory at Woods Hole, Massachusetts, and the Zoölogical Laboratories of Columbia University in New York City. I am indebted to the faculty of Columbia University for their generosity in providing me with research facilities. I am especially indebted to Professor Gary N. Calkins for kindly advice and criticisms and to Ruth R. Kidder for technical assistance.

MATERIAL

Ancistruma mytili is found in great numbers in the mantle cavity of the salt water mussel *Mytilus edulis*. These mussels were collected from the pilings of the wharf at the Bureau of Fisheries at Woods Hole and from the rock breakwaters at Brighton Beach, Manhattan Beach, and Port Washington, Long Island. *A. isseli* is equally abundant in the mantle cavity of the solitary mussel *Modiola modiolus*. This mussel may be found half buried in the muddy flats at low tide. The *Modiola* used in this work were collected just north of the Coast Guard station at Woods Hole. Additional mussels of both genera were taken from Pelham Bay in New York City.

TECHNIQUE

The mussels were brought into the laboratory and placed in large aquaria supplied with running sea water (Woods Hole) or a jet of air (New York). In this situation the ciliates remained alive and in good condition for months, *Ancistruma* proving to be much less sensitive to environmental changes than *Conchophthirius mytili* (Kidder, 1932). The ciliates were obtained for examination by opening the valves of the mussel and washing the contents of the mantle cavity into a Syracuse watch-glass. Under the dissecting microscope individuals were then selected and with a fine pipette placed on coverglasses for examination and fixation.

The fixatives and stains employed were: Schaudinn's, sublimate-acetic, Bouin's, Zenker's and Champy's fluids followed by Heidenhain's and Delafield's hæmatoxylins, the Borrel stain, and the Feulgen nuclear reaction. (The formulæ and methods for both the Borrel stain and the Feulgen reaction may be found in Calkins, 1930.)

The " silver line system " was studied by the silver nitrate impregnation method of Klein (1926*a*, 1926*b*, 1927) and also by a few modifications of it. The most useful modification was to kill the ciliate in osmic acid fumes (10 to 20 seconds). Before the organism became dry a drop of distilled water was added. This was drawn off with a fine pipette, and the process repeated a number of times. Thus the sea salts were removed and the formation of a heavy precipitate, upon addition of the silver nitrate, prevented. A 2 per cent solution of silver nitrate was added and the preparation exposed to the light. The time of impregnation varied with the length of time the organism was exposed to osmic fumes. By watching the process under the dissecting microscope the reaction was halted when the ciliates had reached the desired darkness. The impregnation usually took from one to three hours. The advantage of killing in osmic vapor is that the shape of the ciliate is retained with very little of the distortion that occurs by simply drying.

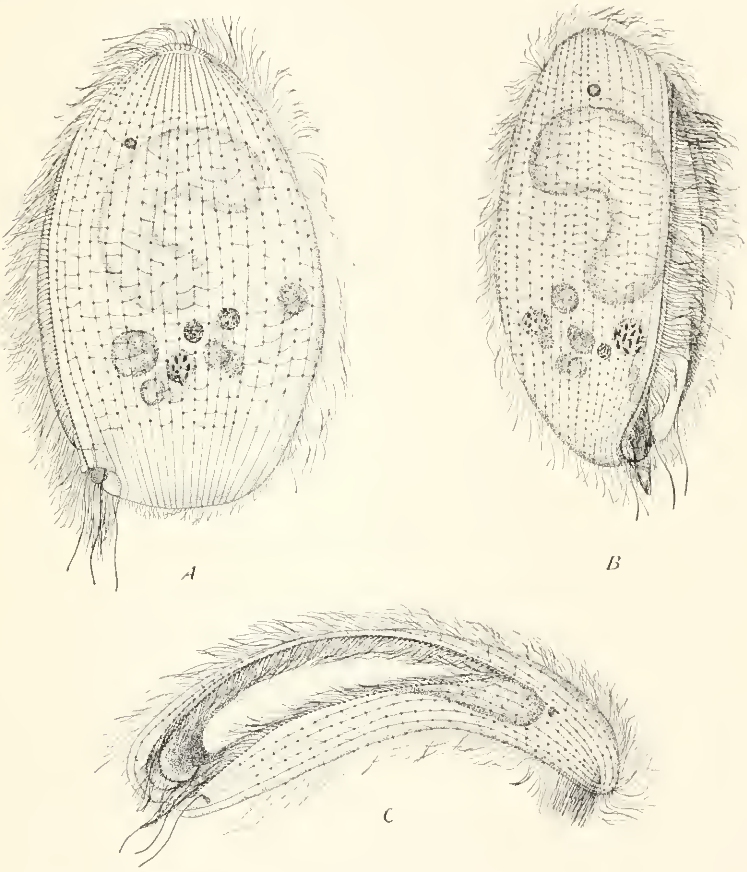

Fig. 1. *Ancistruma mytili.* Camera lucida drawings. × 1000.

A. Ventral view. Schaudinn's: Heidenhain's hæmatoxylin.

B. Dorsal view. Mouth is seen to be slightly dorsal near the posterior end. Zenker's: Mallory triple stain.

C. Lateral view of the right side. At the anterior end may be seen the tuft of straight tactile cilia. Sublimate-acetic: Heidenhain's hæmatoxylin.

For the detailed structure of the ciliary insertions individuals were fixed in Zenker's and Champy's fluids and sectioned. These sections were then stained with Heidenhain's hæmatoxylin or crystal violet-sulph-alizarinate (Benda's alizarin method. Lee's Microtomist's Vade-Mecum, 9th ed., p. 335).

GENERAL FEATURES OF ANCISTRUMA MYTILI

The following terms of orientation will be used in dealing with the members of the genus *Ancistruma*. The end directed forward in normal swimming will be called anterior. The concave surface always in contact with the substrate will be called ventral. (For a discussion of this question, see Kidder, 1932.)

The swimming movements of *A. mytili* are very characteristic. In the Syracuse dish the ciliates continuously leave the bottom of the dish and come to the surface film, then immediately return. During these flights they revolve rapidly on a diagonal axis, and have the appearance of moving in a series of jerky somersaults. They exhibit ceaseless activity and seldom remain at rest for more than a short period of time.

Size and Shape

Ancistruma mytili averages 67 μ (52 μ–74 μ) in length and 31 μ (20 μ–38 μ) in width. These measurements were made on fifty living specimens. Fixed material gives measurements of a considerably lower value. The organism is nearly oval in shape when viewed from the dorsal or ventral surfaces (Fig. 1, *A* and *B*). The left margin is slightly more convex than the right. In lateral view the ciliate is seen to be elliptical and bent into an arc, the ventral surface being deeply concave and the dorsal surface convex (Fig. 1, *C*). The extreme dorsoventral flattening has been accurately described and figured by Maupas (1883) and De Morgan (1925).

The peristomal groove starts on the extreme right margin near the anterior end and extends to the posterior end of the organism. It is narrow and pointed anteriorly and widens gradually toward the posterior end. It terminates in the wide mouth situated in a slightly dorsal position. The dorsal edge of the peristome swings down in an even arc about the mouth. The ventral edge, however, curves sharply to the left in an inverted arc, forming a pointed flap just under the mouth. This flap and the slightly dorsal position of the oral aperture make it exceedingly difficult to study from the ventral surface. The floor of the peristomal groove is thrown into a fold which has the appearance of a tongue and reminds one of the shelf in the peristome of *Conchophthirius mytili* (Kidder, 1932). This tongue extends to the oral aperture, becoming more protruding toward the posterior end (Fig. 1, *B*).

The body of the ciliate is covered with a thick elastic pellicle. No contractility is exhibited, but the whole organism may be bent by mechanical forces.

Peripheral Cilia

The peripheral cilia originate from rather large basal bodies arranged in longitudinal rows. These rows begin at the anterior field slightly back of the anterior extremity on the ventral surface. They pass backward and around the posterior end, returning on the dorsal surface and around the anterior end to the anterior field. The rows on the ventral surface are more numerous and much closer together than those on the dorsal. In sectioned material this relationship is clearly brought out (Fig. 2). The basal bodies are connected longitudinally by fine coördinating fibers.

The cilia are long and wavy with the exception of a single tuft of tactile cilia at the anterior end on the ventral surface. In this region the basal bodies are small and very numerous, and from each originates a single stiff cilium. The tuft of cilia may be seen easily from a lateral view (Fig. 1, *C*). It may also be seen in life when the organism attaches itself to the coverglass ventral side up. Then each cilium appears as a tiny point against the glass while the other cilia over the body continue to wave. This tuft of cilia is the attaching mechanism and is undoubtedly used in anchoring the organism to the mantle and gills of the host. It often attaches itself by this tuft to the bottom of the Syracuse dish or to pieces of debris.

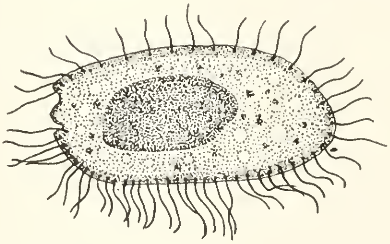

FIG. 2. *Ancistruma mytili.* Camera lucida drawing of a cross-section through the mid region looking from the anterior to the posterior end. Champy's: Crystal-violet. × 1000.

Peristomal Cilia

Slightly back of the pointed end of the peristomal groove three rows of very long cilia originate. Two rows follow the dorsal edge of the peristome posteriorly and dip down in an arc behind the mouth. The other follows the ventral edge of the peristome and ends just over the point of the ventral flap. The cilia of these three peristomal rows are nearly twice as long as the peripheral cilia and are set exceedingly close

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together. When the organism is in motion they are seen to move in waves like undulating membranes. At the posterior ends of these rows arise three still longer and heavier cilia. De Morgan (1925) figures and describes these three cilia. His observations of posterior lobes or dorsal ridges, however, I have been unable to verify in my material although I have looked for them repeatedly. The surface of the organism often does become folded and the lateral edges papillose after remaining on a slide for a short time.

Fibrillar System

The longitudinal coördinating fibers connecting the basal bodies are easily seen after Heidenhain's hæmatoxylin or Mallory's triple stain, but it is with reduced silver that they stand out with great clearness. Successful preparations of the silver line system are relatively easy to obtain using *Ancistruma* as material. The cytoplasm is yellowish and the basal bodies and fibers are black. I have found this method very useful in studying the fine details of the general morphology of this genus in addition to bringing out a system of fine fibers not demonstrated by any other technique.

The fibers are of three types in both *Ancistruma mytili* and *A. isseli:* the longitudinal coördinating fibers, the transverse or commissural fibers, and the net of fine fibers in the peristomal region (Fig. 3, *A*, *B*, and *C*).

The longitudinal fibers of *A. mytili* originate in a clear, bar-like area in the ventral anterior region, the anterior field (Fig. 3, *A* and *C*). This area does not appear to be fibrillary, as it remains unstained after all the methods I have employed. The basal bodies from which the stiff tactile cilia arise are small and very close together. Posterior to this region the basal bodies are larger and farther apart. The basal bodies of the three peristomal rows of cilia are large and numerous.

The transverse fibers are delicate and only occasionally can be seen after hæmatoxylin stain. They stand out quite clearly after silver impregnation, however. They are very irregular in their distribution. I have never seen any in the region of the tactile cilia and only occasional pairs of basal bodies in the posterior region show cross connections. They are most numerous in the mid region of the body on both dorsal and ventral surfaces.

Figure 3, *A*, represents an organism in which the impregnation was exceedingly clear and delicate. In one region on the left side of the organism a few fibers are seen lying between the rows of basal bodies. These may represent the interstrial fibers described by Pickard (1927) in *Boveria teredinidi*. On the other hand, they may represent a deep-lying network of the same type regularly seen in the peristomal region,

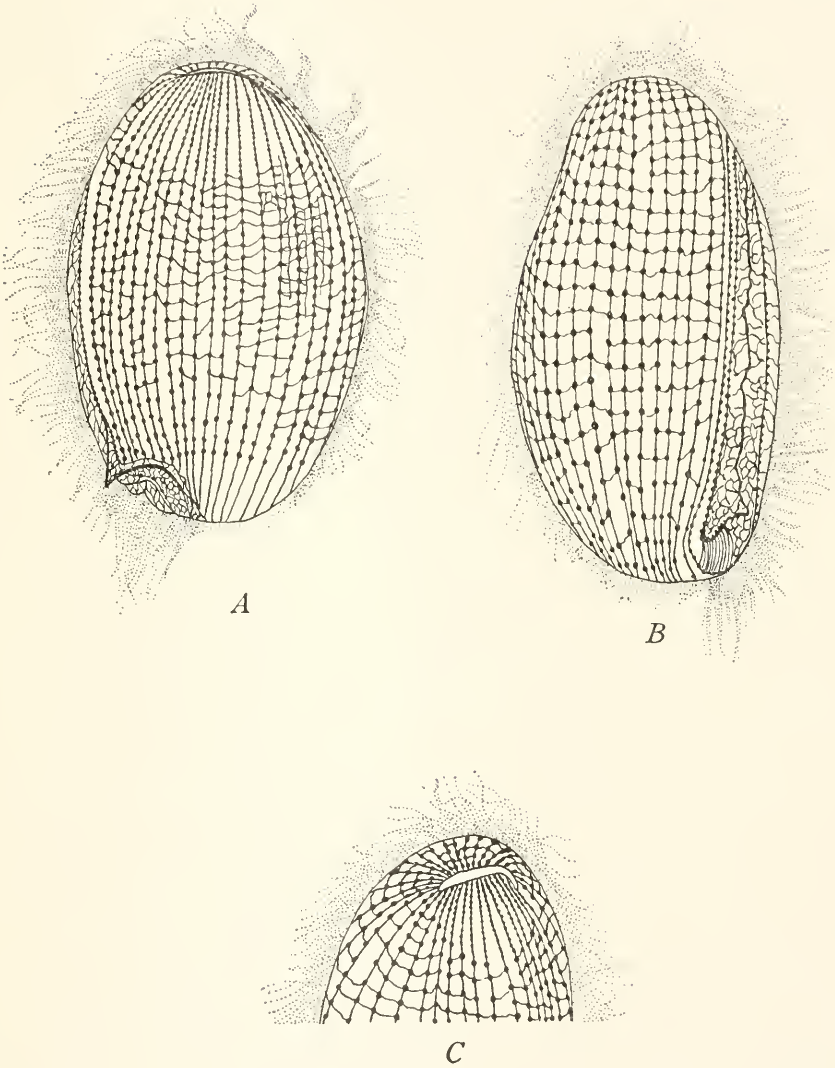

Fig. 3. *Ancistruma mytili.* Camera lucida drawings of organisms impregnated with silver nitrate. × 1000.

A. Ventral view. Note the fine interstrial (?) fibers appearing on the left side of the organism.

B. Dorsal view. The net of fibers in the peristome are readily seen in this view. Note also the row of fine fibers below the mouth.

C. Anterior end of an organism turned slightly on its right side. Note bar-like anterior field.

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their presence being masked by the heavy pellicle in the body region so that they appear only under exceedingly favorable conditions.

The whole peristomal region, even into the mouth, is supplied with a net of very fine fibers. These fibers spread in all directions and seem to connect directly with the basal bodies of the peristomal cilia.

A number of fine fibers connecting the inner dorsal row of peristomal cilia to the outer dorsal row may be seen just posterior to the mouth. They seem to be quite distinct from the net system and probably are in the nature of concentrated transverse or commissural fibers (Fig. 3, *B*).

Nuclei

Ancistruma mytili contains one very small micronucleus located well up in the anterior end. It is spherical and, in the interphase, always stains intensely with any of the basic dyes. It is readily observed as the cytoplasm in the anterior region is very clear and free from food particles.

The macronucleus is relatively huge and very characteristic in shape and position. It is clearly visible in the living organism. It resembles a large, curved sausage, having its convex side always toward the peristome. In some organisms the degree of curvature is less than in others. Maupas (1883) and De Morgan (1925) both sketch the macronucleus of *A. mytili* entirely too small in proportion to the size of the organism. Moreover, De Morgan represented the convex surface as directed toward the left side instead of toward the peristome. I have never found this to be the case in my material.

The macronucleus of *A. mytili* in the resting stage is seen to be composed of very compactly placed chromatin granules. These take the Feulgen reaction intensely and stain clearly with any of the basic dyes.

Vacuoles

Many food vacuoles are found in the posterior portion of the body. These are filled with bacteria and small particles taken from the water brought in by the mussel. Many of the vacuoles contain algæ.

There is one contractile vacuole usually situated well to the left side near the posterior end. It is quite regular in its contractions, filling slowly and emptying rapidly. I have never been able to detect any definite and persistent pore in the periplast through which this discharge takes place. As the organism becomes enfeebled on the slide the rate of discharge decreases until finally it ceases and the vacuole reaches enormous proportions. This persists for one to three minutes before cytolysis takes place.

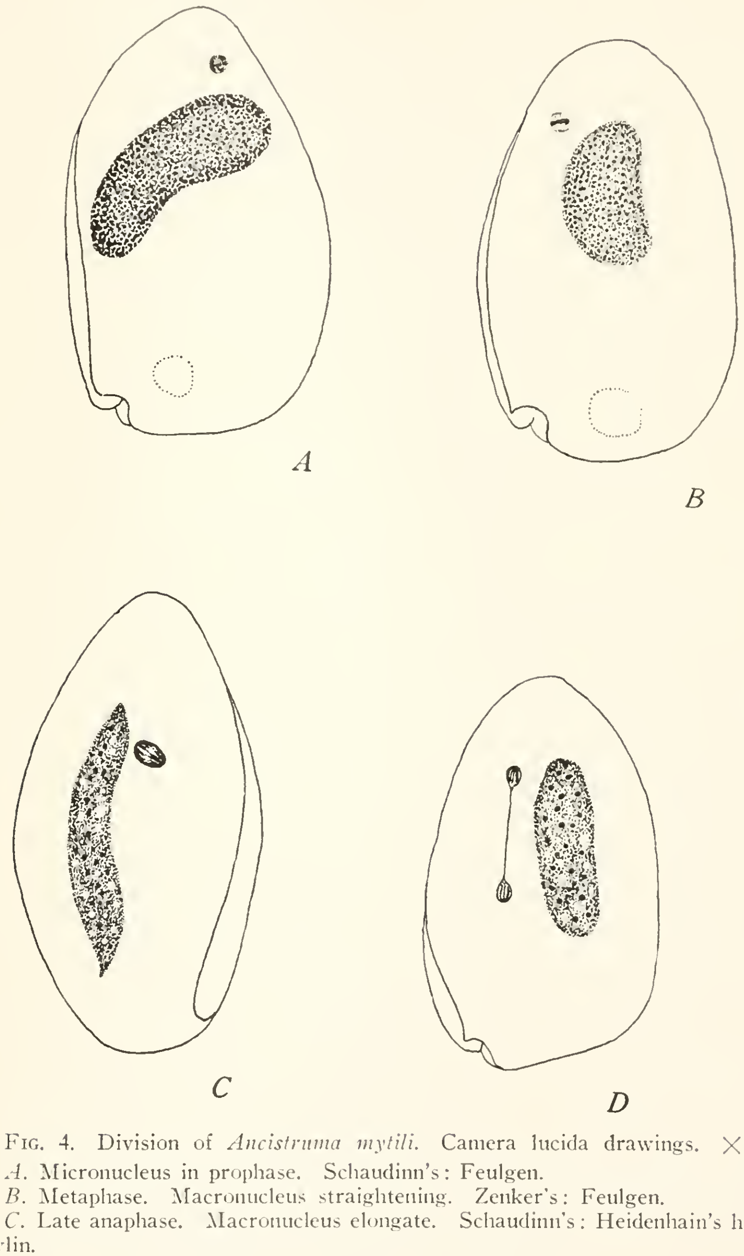

Fig. 4. Division of *Ancistruma mytili.* Camera lucida drawings. × 1000.

A. Micronucleus in prophase. Schaudinn's: Feulgen.

B. Metaphase. Macronucleus straightening. Zenker's: Feulgen.

C. Late anaphase. Macronucleus elongate. Schaudinn's: Heidenhain's hæmatoxylin.

D. Telophase. Schaudinn's: Heidenhain's hæmatoxylin.

Division of Ancistruma mytili

The division of *A. mytili* appears to be regular and of the usual ciliate type. The micronucleus is too small to allow detailed observation of inner structure. I shall outline the process briefly.

The micronucleus initiates fission. Its chromatin becomes less dense and appears as masses in a lightly-staining matrix. The macronucleus straightens out with its long axis corresponding to the long axis of the body (Fig. 4, *A*). There is very little swelling of the micronucleus and the metaphase is formed by an apparent condensation of chromatin into a plate (Fig. 4, *B*). This plate of chromatin divides and the two halves move to opposite poles of the then elongated micronuclear spindle (Fig. 4, *C*). The macronucleus lengthens considerably and becomes filled with deeply-staining granules and clear areas. This reminds one of the condition of the macronuclei of *Uroleptus halseyi* (Calkins, 1930). The micronucleus now divides, pulling out a long connecting strand between the two halves (Fig. 4, *D*). The connecting strand disappears and the two daughter micronuclei migrate to opposite ends of the cell. The macronucleus, meanwhile, begins to constrict and pull into a dumbbell shape (Fig. 5, *A* and *B*). In the clearer area between the dividing halves many large granules are regularly seen (Fig. 5, *B*), but as the separation proceeds these granules of chromatin are drawn up into the daughter halves. The separation of the daughter halves is clean, and the daughter macronuclei round up and become compact. The large granules and vacuoles disappear, and the ciliate undergoes plasmotomy (Fig. 5, *C*). The macronuclei of the daughter ciliates then elongate and assume their characteristic sausage-like shape.

General Features of Ancistruma isseli

A Syracuse dish containing the contents of the mantle cavity of *Modiola modiolus* is a scene of chaos, when viewed through the dissecting microscope. *Ancistruma isseli* exhibits more speed in swimming than does *A. mytili*. The ciliates are exceedingly numerous in most mussels and tend to swim in one direction, in contrast to the jerky flights of *A. mytili*.

Size and Shape

Fifty specimens of *Ancistruma isseli* gave an average length of 77 μ (70 μ–88 μ) while the average width was 42 μ (31 μ–54 μ). These averages are higher than those obtained for *A. mytili* although the ranges overlap.

The shape of *A. isseli* is roughly similar to that of *A. mytili*, but the former is more pointed at both the anterior and posterior ends. The

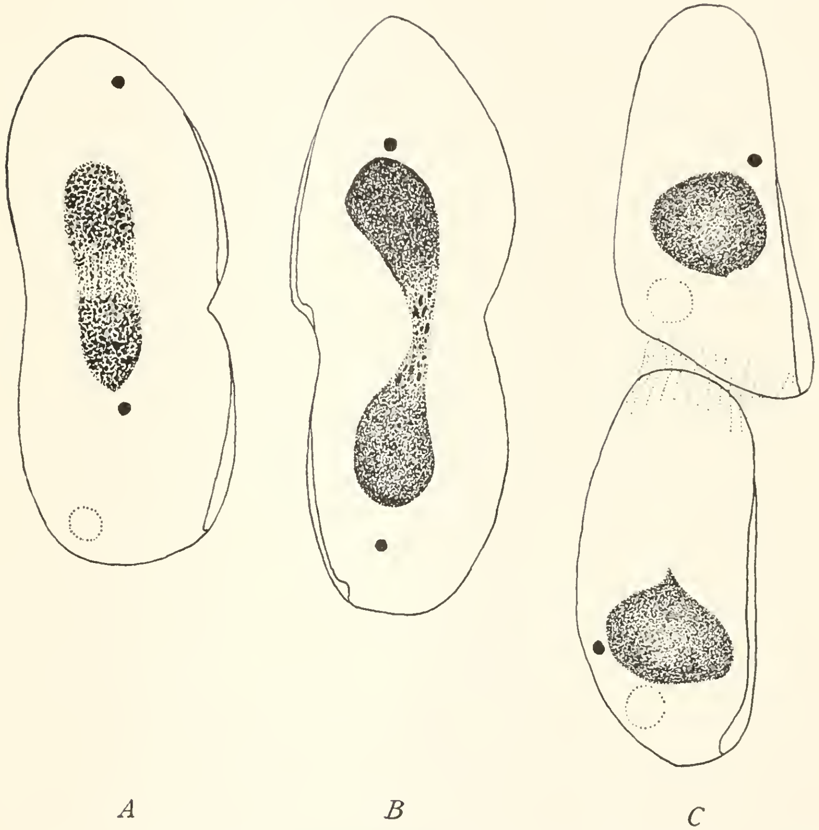

FIG. 5. Division of *Ancistruma mytili.* Camera lucida drawings. × 1000.

A. Daughter micronuclei are again compact and have moved apart. Macronucleus showing division plane. Reorganized peristomes visible. Zenker's: Feulgen.

B. A little later. Macronucleus dumb-bell-shaped. Schaudinn's: Delafield's hæmatoxylin.

C. Division of ciliate nearly completed.

right margin is only slightly convex while the left margin is greatly convex, the widest point being in the posterior third of the body (Figs. 6 and 7).

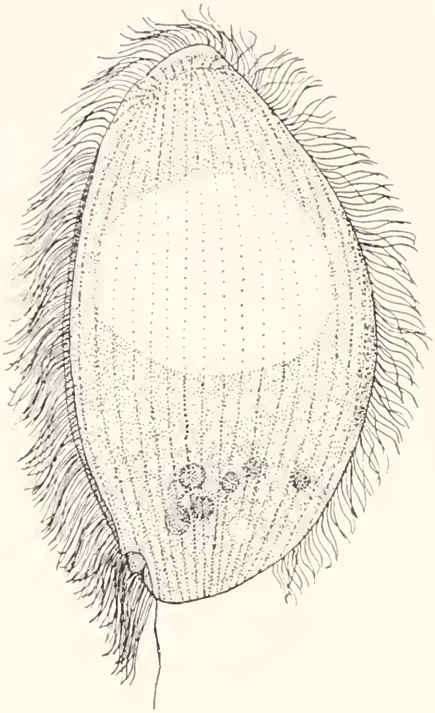

FIG. 6. *Ancistruma isseli.* Ventral view of organism drawn from life at approximately 1000 diameters. The macronucleus is extremely large and clear.

In lateral view *A. isseli* and *A. mytili* resemble one another closely. Both are dorso-ventrally flattened, concave ventrally, convex dorsally, and both possess attaching cilia. *A. isseli,* however, possesses a much enlarged anterior field, at the extreme anterior end. This field is in the form of a clear bubble in the periplast and is seen especially well when the ciliate is flattened under a coverglass. It is usually visible in fixed specimens (Fig. 8, *A* and *B*). The peristomes of the two species are very similar, the pointed flap under the mouth being less prominent, however, in *A. isseli.*

Cilia and Fibrillar System

The peripheral and peristomal cilia of *A. isseli* are long and wavy as in *A. mytili.* The tactile tuft is much less prominent and less extensive in the former species. The ciliary lines do not pass completely about the posterior end but center at an easily visible suture. This suture is only observed from the dorsal side in organisms that are slightly flattened. After silver impregnation it appears as a black fiber (Fig. 8, *B*).

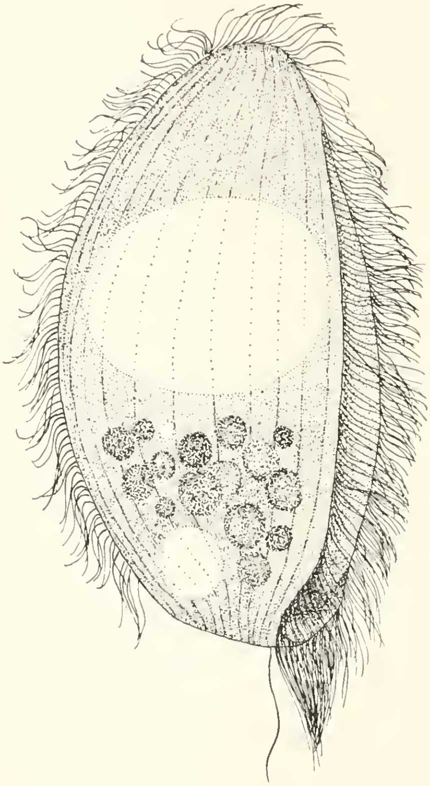

FIG. 7. *Ancistruma isseli.* Dorsal view of living organism at approximately 1000 diameters. Mouth, contractile vacuole and macronucleus visible.

The network of fibers in the peristomal region is much coarser and less extensive than in *A. mytili* and I have never observed any fibers (interstrial?) between the lines of cilia on the body surface. The fine fibrillar connections between the outer and inner dorsal rows of peristomal cilia seem to be lacking in *A. isseli.* Instead the outer dorsal peristomal fiber dips down and forward to join the end of the inner dorsal peristomal fiber just ventral to the mouth.

The fibers of the ciliary system seem to be all interconnected in *A. isseli* through the peristomal net system. The whole fibrillar organization is certainly very similar to that described by MacLennan and Connell (1931) for *Eupoterion pernix*.

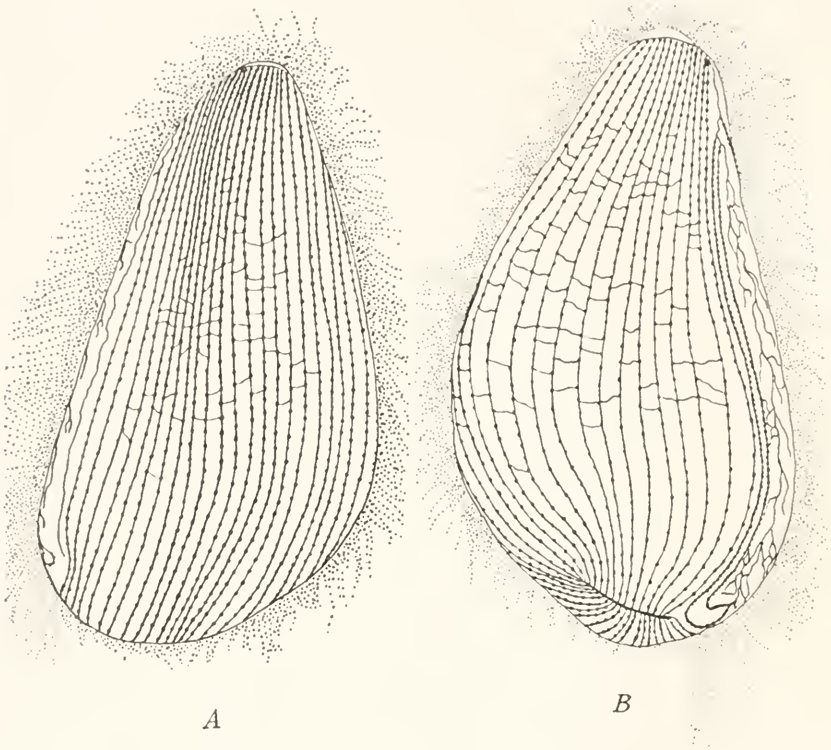

FIG. 8. *Ancistruma isseli.* Silver nitrate impregnations. Camera lucida drawings. × 1000.

A. Ventral view.

B. Dorsal view showing posterior suture. Note also the clear bubble-like anterior field.

Nuclei and Vacuoles

The micronucleus of *A. isseli* is a small sphere situated in the anterior fourth of the body. It is identical with the micronucleus of *A. mytili* as far as can be determined. The macronucleus of *A. isseli* is very distinctive, however. It is clearly visible in life as an enormous sphere occupying the center of the body (Figs. 6 and 7). Some shrinkage in the size occurs upon fixation. The macronucleus is of the massive type but it possesses many spherical deeply-staining chromatin granules among the smaller packed granules (Fig. 9, *A*).

Issel (1903) reports the macronucleus to be fragmented at times into two to seven parts. I have evidence that the spheres so often found in both *Ancistruma isseli* and *A. mytili* are not the result of fragmentation but the reorganization of an ex-conjugant. I shall present this evidence with a discussion of the question in the second of this series of papers.

The food vacuoles are numerous and situated in the posterior third of the body. They are usually filled with the yellow, granular pigment of *Modiola*.

The contractile vacuole is identical in size and position with the contractile vacuole of *A. mytili*.

Division of Ancistruma isseli

The micronucleus of *A. isseli* in division resembles that of *A. mytili*. It is too small to observe with certainty the details of any internal structures. The general sequence of its activity may be seen in Figs. 9, *A*, *B*, *C*, *D*, *E*, *F*, and 10, *A*, *B*, and *C*.

The interesting feature of division is to be found in the macronucleus. As the micronucleus is dividing the macronucleus pulls out into an elongate mass. As this mass begins its constriction two light areas may be seen appearing on either side of the center. These areas are to become the planes of fission of the macronucleus, the heavily-staining mass between them becoming extrusion chromatin (Fig. 9, *D* and *E*). As the two constrictions increase the extrusion mass becomes spherical (Fig. 9, *F*). The two daughter macronuclei now draw apart and round up, pulling out the macronuclear membrane into a long tube flared at the center to accommodate the residual chromatin (Fig. 10, *A*). Very soon the connections between the daughter nuclei are severed and the residual mass contracts (Fig. 10, *B*). The daughter macronuclei round up and plasmotomy proceeds. The residual mass, meanwhile, becomes very loosely granular and loses its staining capacity. By the time the daughter organisms are ready to separate this mass, now in the cytoplasm of one of the daughter organisms, has nearly disappeared (Fig. 10, *C*).

This type of macronuclear action almost exactly parallels that found in *Conchophthirius mytili* (Kidder, 1932) and is quite different from that of *Ancistruma mytili*, described above. The relative amount of chromatin cast out at each division is more than in *Conchophthirius*, but the method of disintegration and absorption into the cytoplasm is the same.

Discussion

Issel's (1903) description of a commensal of *Modiola barbata* under the name of *Ancistrum mytili* was not wholly satisfactory to him. He states that he had some doubts about its identity with the *Ancistrum mytili* of Maupas, mainly because of the spherical macronucleus. But after examining some ciliates from a number of *Mytilus edulis* (obtained from a merchant) he found all gradations from the sphere to the bent type described by Maupas. He attributed this bent appearance to the beginning of fragmentation.

Kahl (1931) recognizes the distinct difference between the two types and names the ciliate from *Modiola barbata, Ancistruma isseli.* From the present study I think it possible to establish the correctness of Kahl's statements, and the validity of his species, as I believe the ciliate here described from *Modiola modiolus* to be identical with the ciliate from *Modiola barbata.*

The main points of difference between *Ancistruma mytili* and *A. isseli* are as follows: (1) Size: *A. isseli*—average 77 μ × 42 μ; *A. mytili*—average 67 μ × 31 μ. (2) Macronucleus: *A. isseli*—spherical; *A. mytili*—sausage-shaped. (3) *A. isseli* has posterior suture; *A. mytili* has no posterior suture. (4) Division: *A. isseli* casts out a mass of macronuclear chromatin at division; *A. mytili* divides sharply with no chromatin extrusion.

The genus *Ancistruma* shows many affinities to the Conchophthiridæ. The peristome of *Ancistruma* is like *Conchophthirius mytili* in that it possesses a shelf or raised portion which protrudes between the rows of peristomal cilia. The motor organization is similar to that of *Eupoterion* as described by MacLennan and Connell (1931). In fact, the main distinctive difference between the members of the Conchophthiridæ (*Conchophthirius, Cryptochilum,* and *Eupoterion*) and the genus *Ancistruma* is the possession of the tactile tuft of cilia by the latter.

The "silver line system" first described by Klein (1926) was thought by him to represent a system of nervous connections and coördinating controls. It is evident from the present study that fibers generally thought to be coördinating or conductive in function (longi-

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FIG. 9. Division of *Ancistruma isseli.* Camera lucida drawings. × 1000.

A. Interphase showing compact micronucleus and large spherical macronucleus. Sublimate-acetic: Heidenhain's hæmatoxylin.

B. Micronucleus in metaphase of mitosis. Sublimate-acetic: Heidenhain's hæmatoxylin.

C. Telophase. Sublimate-acetic: Feulgen.

D. Daughter micronuclei drawing apart. Macronucleus elongating. Sublimate-acetic: Heidenhain's hæmatoxylin.

E. Later stage. Note two constrictions in the macronucleus. These represent the division planes while central mass is the residual chromatin. Gilson-Carnoy's: Borrel.

F. Later stage in division of macronucleus. Gilson-Carnoy's: Feulgen.

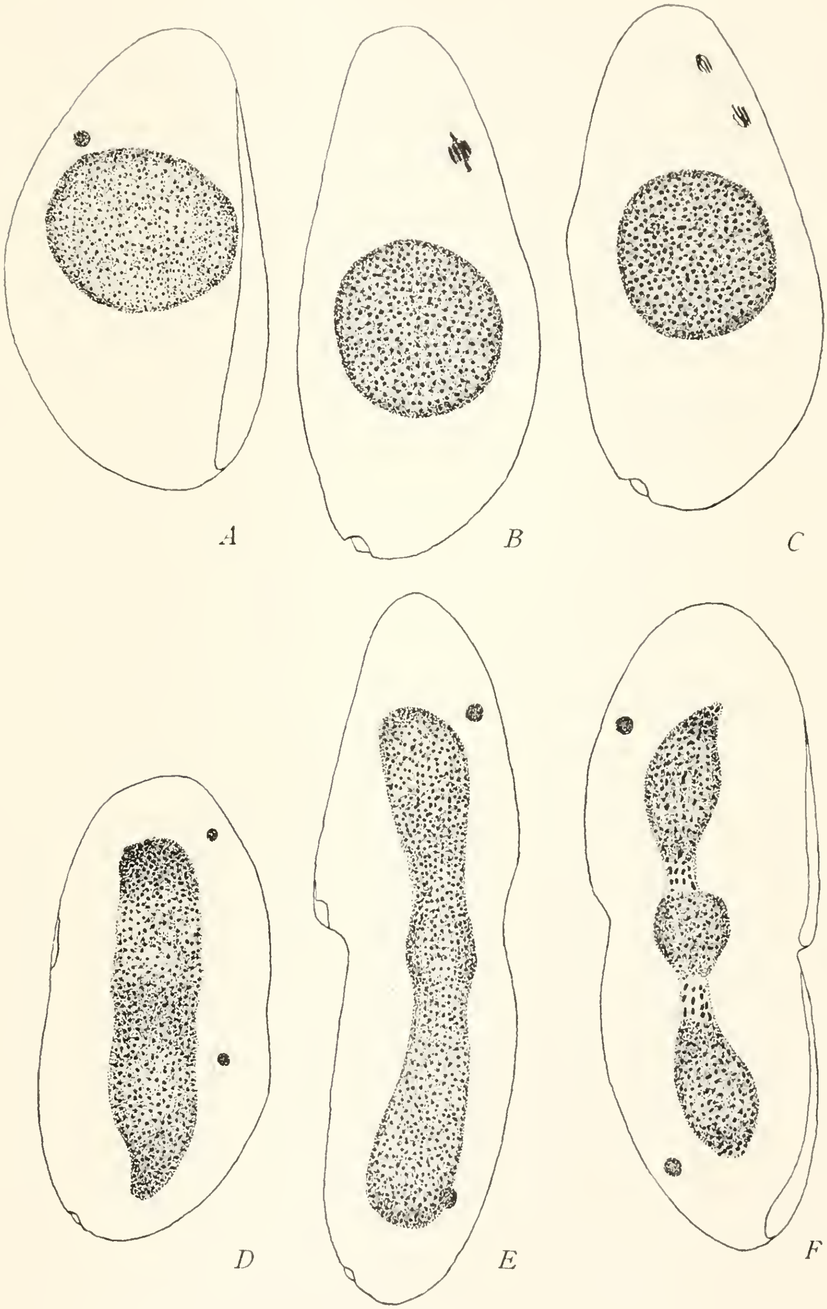

Figure 9

tudinal fibers) are stained by the reduced silver after this method. It seems logical to suppose that the network of fine fibers in the peristomal region of *Ancistruma* is conductive and serves to connect this sensitive area with the organelles of locomotion.

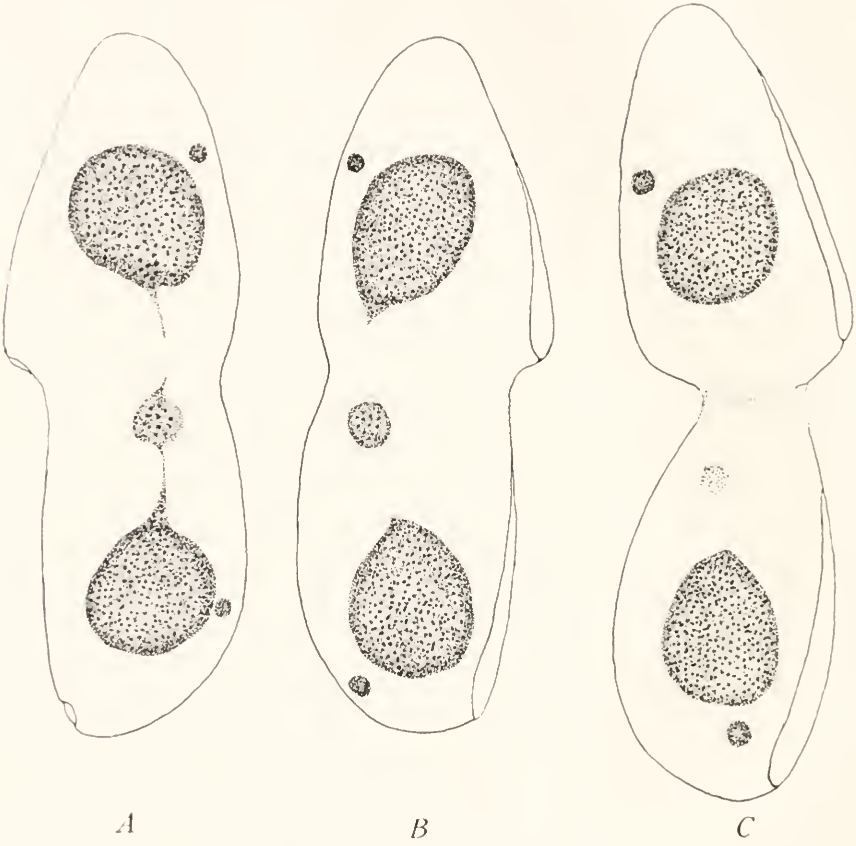

FIG. 10. Division of *Ancistruma isseli.* Camera lucida drawings. × 1000.

A. Late division of macronucleus. Extrusion chromatin still within macronuclear membrane. Gilson-Carnoy's: Borrel.

B. Later. Daughter macronuclei separate. Residual mass of chromatin free in cytoplasm. Sublimate-acetic: Feulgen.

C. Daughter macronuclei rounding up. The residual chromatin is being absorbed into the cytoplasm of posterior daughter organism. Sublimate-acetic: Feulgen.

It seems strange that two such similar forms as *Ancistruma mytili* and *A. isseli* should show such a decided difference in macronuclear division. If this chromatin elimination is a purification process as suggested by Calkins (1930), then there must be some fundamental difference between the two species in the chromatin reorganization during division. *Ancistruma mytili* must be able to eliminate its nuclear waste products gradually or at least in a manner as yet impossible of visual detection. Whether or not this process of chromatin elimination has any phylogenetic significance is unknown. Perhaps some light may be thrown on this question when sufficient data have been collected and analyzed. The Conchophthiridæ and the Ancistridæ seem to be the most favorable material for such a study.

SUMMARY

1. *Ancistruma mytili* Quenn., a holotrichous ciliate commensal in the salt water mussel *Mytilus edulis*, and *Ancistruma isseli* Kahl, a holotrichous ciliate commensal in the solitary mussel *Modiola modiolus* are described.

2. Observations are made from both living and fixed material.

3. The motor system of each consists of long peripheral cilia, a tuft of tactile cilia, and three rows of peristomal cilia all supplied with interconnected basal bodies. Net-like systems of coördinating fibers occur in the peristomal regions.

4. Each species possesses one micronucleus, which is small and compact.

5. The macronucleus of *A. mytili* is sausage-shaped while that of *A. isseli* is spherical. Both are very large.

6. During division the micronuclei divide in the usual manner. They are too small to allow observation of detail.

7. The macronucleus of *A. mytili* divides evenly and cleanly, while that of *A. isseli* eliminates a large ball of chromatin at each division.

8. The differences between the two species are discussed.

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GRUBERIA CALKINSI SP. NOV., A BRACKISH-WATER CILIATE (PROTOZOA, HETEROTRICHIDA) FROM WOODS HOLE, MASSACHUSETTS

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University of Mexico

While working at the Marine Biological Laboratory, Woods Hole, Mass., last summer, I had the opportunity to collect many interesting forms of Protozoa from a small brackish-water deposit situated near Gardiner Road, locally known as " Lillie's Ditch."

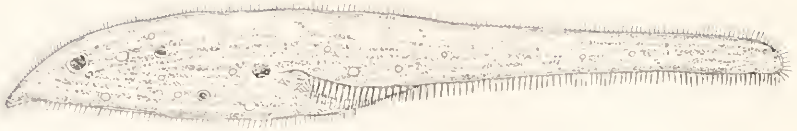
Among those forms studied I found a large ciliate belonging to the family Plagiotomidæ, exceptionally abundant during August and September. At that time I was unable to place it in any of the genera previously described.

As may be seen (Fig. 1) this animal has a superficial similarity to *Spirostomum*. It differs, however, in some important respects, particularly in the possession of many vacuoles (possibly contractile) which are scattered throughout the body, instead of the characteristic large posterior contractile vacuole of *Spirostomum*, as seen in the typical species.

Gruber (1884) described a new species of *Spirostomum* (*Sp. lanceolatum*) while studying the Protozoa from the port of Genoa, Italy. Gruber's form presents striking similarity to the animals which I collected from Lillie's Ditch, but differs mostly in respect to the morphology of the nucleus. It also lacks the characteristic posterior contractile vacuole of *Spirostomum*.

Some years ago De Morgan (1923–1925) found in the sea-water tanks at Plymouth Laboratory some specimens of *Spirostomum lanceolatum*. His description, far more accurate and complete than that offered by Gruber (1884), shows some points of difference from the latter and a closer similarity in nuclear structure to the animals found at Woods Hole. However, the differences are sufficient to separate perfectly the two species.

Florentin (1899) described under the name of *Spirostomum salinarum* a brackish-water form, which he found in Lorraine, France, as having a yellow color, non-contractile body, and living in media of high saline concentration. The presence of small, independent, granular nuclei, and a typical posterior contractile vacuole, are characters which



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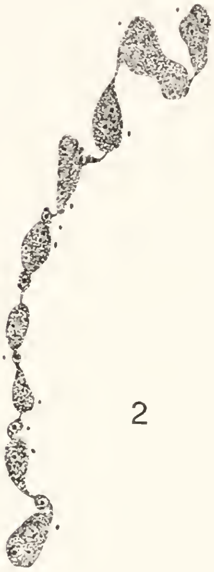

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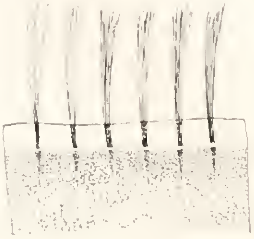

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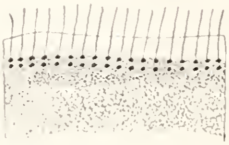

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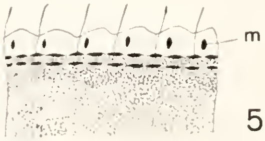

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FIG. 1. *Gruberia Calkinsi* sp. nov. From a living specimen.

FIG. 2. Nuclear apparatus. Hg_2Cl, Heidenhain's hematoxylin.

$$\frac{\text{Zeiss Comp. } 15\times}{\text{Zeiss Apo. } 90\times,\ 1.3\text{ n. a.}}.$$

FIG. 3. Longitudinal section (5 microns thick) showing membranelles. Hg_2Cl, Heidenhain's hematoxylin.

$$\frac{\text{Zeiss Comp. } 15\times}{\text{Zeiss Apo. } 90\times,\ 1.3\text{ n. a.}}.$$

FIG. 4. Longitudinal section (5 microns thick) of the periplast, showing ciliary arrangement. Hg_2Cl, Heidenhain's hematoxylin.

$$\frac{\text{Zeiss Comp. } 15\times}{\text{Zeiss Apo. } 90\times,\ 1.3\text{ n. a.}}.$$

FIG. 5. Transverse section (5 microns thick) of the periplast; *m*, myonemes. Hg_2Cl, Heidenhain's hematoxylin.

$$\frac{\text{Zeiss Comp. } 15\times}{\text{Zeiss Apo. } 90\times,\ 1.3\text{ n. a.}}.$$

are quite sufficient to differentiate this species from the Woods Hole animals.

After careful consideration, I reached the conclusion that I had obtained a new genus of the family Plagiotomidæ.

Three weeks ago, Professor Gary N. Calkins was kind enough to call to my attention the last fascicle (1932) of Kahl's monumental work on the Infusoria. This book has just come out of press, and presents a consideration of the Heterotrichida. This author separates the animal described by Gruber (1884) from the genus *Spirostomum* and creates the new genus *Gruberia* for it, and another species which he calls *Gr. uninucleata.* He also transfers Florentin's *Spirostomum salinarum* to the genus *Blepharisma.* I fully subscribe to these changes, which I think are justified.

The animal which I have studied differs in some respects from those described by Kahl in his genus *Gruberia,* and, evidently, is not either of the species he mentioned. However, the differences are not sufficiently strong to create a new genus, and I prefer to describe the animal found at Woods Hole as a species of the genus *Gruberia.* It has been a great pleasure for me to name it *Gruberia Calkinsi,* as a manifestation of my indebtedness to Dr. G. N. Calkins, not only for his invaluable teachings but also for the kindly interest he has taken in my work, and the many attentions he has shown me during my stay at Woods Hole and Columbia University.

Description

The size varies from 200 to 800 microns in length. The width ranges from one-fifth to one-seventh of the total length.

The animal (Fig. 1) is long and straight, with an obtuse anterior end that is slightly curved toward the ventral side. The posterior end is more or less acutely pointed, and is frequently curved in the same direction as the anterior end.

This general shape is maintained within certain limits. The body is slightly contractile, but the general shape is not altered when contraction occurs. However, some animals may be obliquely twisted on their longitudinal axes.

The peristomial groove is quite long and extends posteriorly in a straight line to two-thirds of the total body length. It is a relatively deep and broad furrow that gradually sinks into the body. On one margin of the peristome there is a line of membranelles, and on the opposite margin is a row of cilia, which are longer than those which may be found in other parts of the body. Sometimes the posterior portion of the peristome may be obliquely twisted to one side.

The mouth, which is fairly wide, leads into a short and narrow pharynx. The membranelles of the peristome continue into the mouth and form a complete spiral within the gullet. This condition is quite similar to that which has been described by Fauré-Fremiet (1912) in *Condylostoma patens.*

The cilia are small and fine, and are arranged in longitudinal rows which extend over the entire length of the body, in lines which follow the general shape of the animal. There are many small vacuoles (possibly contractile) irregularly distributed throughout the body, but they are generally more abundant in the posterior part of the animal.

The macronucleus is of the beaded type, and is situated on the dorsal side of the body. It possesses generally about ten beads.

There are many micronuclei. These are small and irregularly distributed near the beads of the macronucleus, which they generally exceed in number.

I collected all my animals from Lillie's Ditch, in which place occasionally they were exceedingly abundant.

Professor D. H. Wenrich, of the University of Pennsylvania, has shown me some specimens which are apparently the same animal that I have studied. These were collected by him in the Eel Pond, Woods Hole, Mass.

STRUCTURE

My intention is not to make a detailed cytological study of these animals. However, the careful observation of a great number of living and fixed specimens permits me to describe some of their structural details.

Nuclear Apparatus

The macronucleus (Fig. 2) quite markedly resembles that of *Spirostomum ambiguum.* It extends lengthwise through the whole body, excepting the anterior and posterior ends. It is situated dorsally, and immediately below the cortical layer.

In the living specimens the nucleus is easily seen as a series of clear spaces. After treatment with dilute aqueous solution of neutral red, the macronucleus appeared to be enclosed within a very fine membrane. This membrane stains less intensely than do the beads. Each bead is formed of a densely and finely granulated substance, in which lie some larger and more refringent granules.

Slides which were fixed with a saturated solution of mercuric bichloride in 95 per cent alcohol, and stained with Heidenhain's iron hematoxylin, or Kornhauser's modification, show the beads to be composed of a finely granular groundwork in which are dispersed larger, deeply-staining granules, which correspond to the refringent granules mentioned above.

The connecting membrane is clear and definite, but generally does not stain very darkly. This membrane may be likened to a sack which bulges in places wherein the macronuclear beads are contained. The connecting portion between two beads may be narrow and clear, or may contain a deeply-staining spherical granule, which is generally larger than those which are found in the beads.

With the Feulgen nuclear reaction, large vacuoles appear in the granular background of the beads. These vacuoles occupy the same position in the bead as the large refringent granules that were demonstrated by staining with neutral red and the large, deeply-staining granules seen in specimens that were stained with iron hematoxylin.

I have not been able to find a single dividing animal, although I have observed more than one hundred individuals. For this reason I cannot treat of the behavior of these granules during nuclear division.

However, the fact that these granules, which stain with iron hematoxylin, do not appear in specimens that have undergone the Feulgen reaction, and the fact that their number and size vary greatly, have led me to believe that they may be homologous with what Calkins (1930) calls " X-granules." The small Feulgen and hematoxylin-staining granules in the macronuclear beads may be homologous with the " C-granules " described in *Uroleptus Halseyi* by the same author.

The micronuclei are generally no more than one or one and a half microns in diameter (a single bead of the macronucleus measures from 10 to 40 microns on its major axis). They are scattered near the macronucleus beads, but may sometimes be imbedded in a surface cavity of the latter.

With Heidenhain's hematoxylin they stain very well, but are never so dark as the supposed X-granules of the macronucleus. The Feulgen reaction demonstrates them as poorly-staining bodies. This probably is an indication of low nucleic acid content.

Membranelles

In the living specimens these structures are narrow, triangular in shape, and situated very close to each other; each measures about 30 microns in length.

In stained specimens, a membranelle is always broken up into its constituent cilia (Fig. 3). It passes through the periplast in the form of a slender, relatively deeply-staining shaft, which emerges into the thin ectoplasmic layer, and there assumes the form of a slender cone, which has its base nearest the periplast. There appears to be a small space which separates the shafts from the cones of the membranelles, and the entire ectoplasmic layer from the periplast. This space may be a result of fixation.

The membranelles are strikingly similar in structure to those described by Maier (1903) in *Stylonychia*.

Cilia

The cilia are small and thin, and are arranged in rows which extend all along the animal's body. In longitudinal sections (Fig. 4) they are uniformly distributed throughout the periplast, and arise from spherical basal granules, which are one micron in diameter. These small bodies form a row immediately below the periplast, which is 2 microns thick. A second row of similar granules appears directly beneath them. As far as I could determine, there was no connection between these two rows.

In transverse sections (Fig. 5), the cilia are more separated than in longitudinal sections (a space of three or four microns exists between two consecutive cilia). They are inserted in the lower parts of the undulating periplast, and have the same relations to the double row of granules as shown above. The granules appear to be fusiform in shape, with their longest axes parallel to the periplast. They are situated very closely to each other, and may be connected by pointed ends.

The same apparent lack of connection between the two rows, as shown in longitudinal sections, appears in transverse sections.

Myonemes

Slender, refringent fibers which extend lengthwise over the body can easily be seen in the animal. These are about one micron in diameter and extend parallel and very close to the ciliary rows. They are distinct at the middle body region, and sometimes anastomose at the ends.

I interpret these fibers as myonemes, although the animal is only slightly contractile. And I suppose that the interciliary bodies, which in transverse sections appear within the hyaline periplast (Fig. 5, *m*), are cross-sections of these fibers.

These fibers are similar in position to the myonemes described by Bovard (1907) in *Condylostoma patens*.

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THE OCCURRENCE OF THE HUMORAL CHROMATOPHORE ACTIVATOR AMONG MARINE CRUSTACEANS

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(From the Mount Desert Island Biological Laboratory and the Zoölogical Laboratory, Rutgers University)

The chromatophore-activating principle of the crustacean eye-stalk has been found in *Palæmonetes* by Perkins (1928), in *Crangon* and several species of *Leander* by Koller (1928), in *Macrobrachium* by Smith (1930), in *Praunus* by Koller and Meyer (1930), and in *Callinectes* by Perkins and Kropp (1932). Already indicated as present in several families of decapods, the hormone was looked for in as great a variety of crustaceans as could be obtained in the vicinity of the Mt. Desert Island Biological Laboratory, at Salisbury Cove, Maine. We had available seven different species of marine crustaceans belonging to as many different families. Six were decapods—*Crago* (= *Crangon*) *boreas, Pandalus montagui, Homarus americanus, Pagurus longicarpus, Libinia emarginata,* and *Cancer irroratus;* one was a schizopod—*Mysis stenolepis.*

The eyes were extracted in sea water as has been described previously. Unfortunately, the extracts could not in all cases be tested on the species from which they were derived. This was impracticable with *Homarus, Libinia* and *Cancer* because of the lack of external chromatophores that undergo readily observable changes. *Pandalus* and *Pagurus* did not remain alive long enough under laboratory conditions to permit adequate tests, and *Mysis* was too small for injection in addition to being very fragile and non-resistant. Therefore, *Crago boreas* was used as a test animal for all extracts. Measured amounts of the extract in question were injected into two sets of *Crago* which had previously been adapted to white and black backgrounds respectively. In all cases the chromatophores of *Crago* were examined microscopically before and after the eye extract was injected in order to determine the degree of contraction or expansion. Control animals, similarly adapted, received like amounts of sea water, and still others received the same amount of the donor's abdominal muscle similarly extracted in sea water. Following injections, the animals were returned to the background on which they had become adapted; *i.e.*, white-adapted *Crago,* injected with eye extract, were returned to the white background, and black-adapted *Crago* to the black background. We also took the opportunity to repeat the experiments of Koller (1928) in which he reports the presence of a chromatophore-expanding material from the rostral region of *Crangon.*

Every extract of eye-stalks tested induced a condition in black-adapted *Crago* indistinguishable from the white-adapted state. The effect on the white-adapted animals was to increase the degree of contraction already present. Control animals, whether treated with sea water or extracts of abdominal muscle, showed no comparable effects and in most cases were wholly unaffected. Regarding the action of the rostral region extract we must report completely negative results, as has been reported by Perkins and Snook (1931). In no instance could we obtain a chromatophore-expanding action following the injection of this extract into white-adapted *Crago* although care was taken to prepare the material from the region designated and in the manner prescribed by Koller.

The results are given in Table I. No standard has yet been devised for the assay of the crustacean eye hormone. Pending such an assay we adopted a procedure in the preparation and use of the extracts which was designed to give extracts that would be broadly comparable in potency. This was not done, however, with the intention of obtaining at this time quantitatively comparable extracts of known strength, since the potency of a given extract is undoubtedly related to the total mass of the eye and stalk which we were not able to consider. The extract was prepared from a known number of eye-stalks and a measured amount of medium in such a way that 1 cc. of the final extract possessed the hormonal potency of 40 eye-stalks. The quantity injected was 0.1 cc., representing 4 eye-stalks. This was found to be well above the minimum dosage necessary to induce chromatophore response in *Crago* regardless of the species from which the eyes came. Indeed Koller (1930) estimates that the hormone is still active in a 1 : 100,000 solution. With the dosage used, the onset of contraction occurred in one minute with *Pagurus* extract and in five minutes with *Homarus* extract. Maximum contraction was reached three to four minutes after injection with *Pagurus* extract, and within ten minutes of injection with *Homarus* extract. The initial black-adapted state reappeared after ten minutes with *Pagurus* extract and after thirty minutes with *Homarus* extract. All other extracts brought on responses within these limits, except *Mysis,* which called forth a response

TABLE I

Donors	Crago boreas	Pandalus montagui	Cancer irroratus	Homarus americanus	Libinia emarginata	Pagurus longicarpus	Mysis stenolepis
Eye-stalk extract							
Black-adapted *Crago*	contraction	contraction	contraction	contraction	contraction	contraction	contraction
White-adapted *Crago*	no effect	no effect	sharp maximal contraction	no effect	no effect	maximal contraction	no effect
Abdominal muscle extract							
Black-adapted *Crago*	no effect	no effect	no effect	no effect	no effect	no effect	no effect
White-adapted *Crago*	no effect	no effect	no effect	no effect	no effect	slight local expansion	no effect
Rostral region extract							
Black-adapted *Crago*	no effect	no effect	no effect	no effect	no effect	slight local contraction	not tested
White-adapted *Crago*	no effect	no effect	no effect	no effect	no effect	slight local contraction	not tested
Sea water							
Black-adapted *Crago*	no effect	no effect	Slight local contraction	no effect	no effect	slight local contraction	no effect
White-adapted *Crago*	no effect	no effect	slight general expansion	no effect	no effect	slight local expansion	no effect

five to six minutes after the injection, maximum contraction occurred after fifteen minutes, and the original black-adapted condition did not supervene until thirty minutes after the injection. The apparently weaker extract from the eye-stalks of *Mysis* may be associated with the presence of a specifically less potent hormone as well as with the greatly inferior mass of the eye-stalk compared with that of the decapods examined.

Table II represents the occurrence of the crustacean eye-hormone as determined to date.

TABLE II

Family	Species	Author
(Decapoda)		
Crangonidæ.............	Crangon vulgaris	Koller (1928)
	Crago (=Crangon) boreas	
Palæmonidæ............	Palæmonetes vulgaris	Perkins (1928)
	Leander adspersus	Koller (1928)
	Leander serratus	Koller (1928)
	Leander squilla	Koller (1928)
	Macrobrachium acanthurus	Smith (1930)
Pandalidæ..............	Pandalus montagui	This paper
Nephropsidæ............	Homarus americanus	This paper
Paguridæ...............	Pagurus longicarpus	This paper
Maiidæ.................	Libinia emarginata	This paper
Cancridæ...............	Cancer irroratus	This paper
Portunidæ..............	Callinectes sapidus	Perkins and Kropp (1932)
(Schizopoda)		
Mysidæ.................	Mysis stenolepis	This paper
	Praunus inermis	Koller and Meyer (1930)
	* Praunus flexuosus	Koller and Meyer (1930)

* Koller and Meyer (1930) report the eye-stalk extract from white-adapted *Praunus flexuosus* as causing slight darkening in white-adapted *Crangon vulgaris*. This reaction is contrary to that induced by eye-stalk extracts of all other crustaceans thus far investigated, and may indicate a greater diversity of action of the hormone within the group than has thus far been anticipated.

The above would indicate that the chromatophore-activating substance of the crustacean eye-stalk is very widely distributed among the members of this group and by no means confined to the decapods. Its occurrence, therefore, cannot be regarded as fortuitous in a single species, but as genetically present probably throughout the group and correlated with the chromatophore effectors in the larval or adult stages.

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THE RELATIVE NUMBERS OF IMMATURE ERYTHROCYTES IN THE CIRCULATING BLOOD OF SEVERAL SPECIES OF MARINE FISHES

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During the course of a study of the reactions of the erythrocytes of fishes to vital dyes (Dawson, 1932) in which brilliant cresyl blue was used to produce patterns of reticulation, it became increasingly obvious that the numbers of immature erythrocytes present in the circulation varied widely in different species. In some fishes the numbers of immature cells were negligible while in others they attained considerable proportions.

These observations were made in the summer of 1931 at the Marine Biological Laboratory on seventeen different species. At that time blood was routinely drawn from the heart. Later it seemed possible that the source of the blood samples might in some measure be responsible for the variations encountered, since in many of the lower vertebrates the final differentiation of a considerable number of erythrocytes commonly takes place in the intertrabecular spaces of the ventricle. Variations in the depth of penetration of the needle might then be suspected as a possible cause of the variations in the number of immature cells, depending on whether the tip of the needle stopped in the intertrabecular spaces or passed into the main channel of the ventricle.

Consequently it was decided to repeat the study, drawing samples of blood from both the caudal vessels and the heart. In the summer of 1932 it was possible to re-study the blood of many forms which had been available in 1931 as well as to extend the study to include several additional species. In no case, however, was the blood from the heart and the caudal vessels found to vary appreciably in the number of immature erythrocytes present. Accordingly, it was concluded that the earlier observations were significant and that the blood of the different species of fishes may show specific differences in the proportions of immature cells present in the circulation.

Information on this subject appears to merit some attention. The erythrocytes of fishes are widely used by physiologists in studies on respiration and hemolysis, and the varying number of immature cells present should be taken into consideration when comparisons of the behavior of the blood of different fishes are made.

MATERIAL AND METHODS

As previously stated, these studies were carried on at Woods Hole in the summers of 1931 and 1932. The blood of the fishes studied in 1931 was drawn only from the heart, while in 1932 samples were taken from both the heart and caudal blood vessels. In many instances it was possible to secure in 1932 specimens of species studied the previous year, but in a few cases some species were not obtainable in the second summer. On the other hand, several additional species were available in 1932. In all, the proportion of immature cells in the circulating blood of twenty species was determined. Freshly-drawn blood was prepared by three different methods. Supravital preparations were made by adding a small drop of blood to a slide previously filmed with a saturated solution of brilliant cresyl blue in absolute alcohol. A circular coverslip was added and the preparation sealed with warm vaseline. In order to secure by this method slides on which accurate counts can be made, it is necessary to add the proper amount of blood, just enough so that when the coverslip is added the blood film will be one cell thick and barely reach the margin of the cover. Preparations which do not fulfill these requirements are unsatisfactory for quantitative studies.

More permanent preparations, also demonstrating the patterns of reticulation, were made by mixing the blood thoroughly with an oxalated solution of brilliant cresyl blue in cold-blooded Ringer's solution (Cook, Meyer and Tureen, 1931). A one per cent solution of brilliant cresyl blue and one per cent solution of potassium oxalate were used in the proportions of one drop of oxalate to five drops of stain. Equal parts of blood and oxalated stain were thoroughly mixed in a capillary pipette, spread thinly on a slide, and stained lightly by Wright's technique. In addition, ordinary smears stained with Wright's were employed to demonstrate the varying degrees of basophilia and polychromasia of the immature cells.

In general it was found that more accurate counts could be made when the patterns of reticulation were adequately developed with brilliant cresyl blue. Corresponding counts of immature cells in blood stained only by Wright's method ran uniformly lower, probably due to the fact that fatigue occurs much more rapidly when the observer has to evaluate continuously the varying degrees of basophilia and polychromasia present. Reticulation patterns, on the other hand, are definite and the amount of reticulation present is much more readily determined.

As shown in an earlier study (Dawson, 1932), remnants of the reticulation patterns persist in practically all the mature erythrocytes of fishes. The degree of persistence varies somewhat in different species and also in the individual erythrocytes of the same species. When erythrocytes of varying degrees of differentiation are present, the distinction between maturity and immaturity must at times be a relatively arbitrary one. In this regard the slides doubly stained with brilliant cresyl blue and Wright's stain proved valuable, since the amount of reticulation and the degree of basophilia, polychromasia, or eosinophilia present in the individual cells could promptly be correlated.

For the purposes of this study the various types of erythrocytes encountered were arbitrarily divided into five classes as follows: (1) Mature erythrocytes, with the reticular material in scattered granules or isolated, irregular filaments and stained orthochromatically with eosin in Wright's; (2) less mature cells with the reticular material forming a loose, partially fragmented reticulum, staining orthochromatically with eosin or exhibiting a slight polychromasia; (3) younger cells with an open-meshed but practically complete reticulum and definitely polychromatic; (4) still younger cells with a close-meshed, complete reticulum exhibiting a decided basophilic tint when differentially stained; (5) young cells with the reticular substance massed in a dense, almost granular pattern, presenting little evidence of the true reticulation pattern. These cells give a definite basophilic reaction but are rarely seen except in the elasmobranchs.

Counts were made under oil immersion at a magnification of 900 diameters. In order to facilitate counting, a small coverslip was hand-ruled in India ink with a fine pen into areas 2.5 mm. square. This was used as an ocular micrometer at the level of the diaphragm. Four such areas could be used in the field of the microscope and their size was such that the number of erythrocytes seen in an individual square was small enough to be counted readily without any uncertainty. In 1931 only one sample was drawn from the heart but in 1932 an additional sample was drawn from the tail of each fish and two supravital preparations of each sample were counted. Twenty fields of four squares each were taken and the results averaged. In practice this meant that about one thousand cells were counted from each sample. These values were also checked by similar counts made on preparations doubly stained with brilliant cresyl blue and Wright's stain and on preparations stained with Wright's alone. The number of fishes of each species examined was not constant and data on this point will be included in the description of the findings.

RESULTS

On the basis of the number of immature erythrocytes present in the circulation, the twenty species of fish fall naturally into four groups.

TABLE I

Counts of the several classes of erythrocytes present in the circulation of twenty species of fishes studied at Woods Hole during 1931 and 1932. Only the averages are given. Counts are recorded as "few" when no more than ten were encountered in two supravital preparations and "rare" when less than five were seen.

Groups	Species	Number of Individuals 1931	Number of Individuals 1932	I Isolated Granules and Filaments	II Fragmented Reticulum	III Open-meshed Reticulum	IV Close-meshed Reticulum	V Dense Granular Reticulum
				per cent	*per cent*	*per cent*	*per cent*	*per cent*
I	Mackerel, *Scomber scombrus* Linnaeus	4	2	83.8	3.3	4.3	8.6	0.0
	Menhaden, *Brevoortia tyrannus* (Latrobe)	4	2	83.5	3.0	2.4	11.1	0.0
	Alewife, *Pomolobus pseudoharengus* (Wilson)	4	0	83.3	3.6	2.2	10.9	0.0
	Summer Herring, *Pomolobus æstivalis* (Mitchill)	0	2	79.2	6.5	4.1	10.2	0.0
	Bluefish, *Pomatomus saltatrix* (Linnaeus)	0	2	84.7	5.9	3.2	6.2	0.0
	Common Eel, *Anguilla rostrata* (Le Sueur)	4	3	77.4	4.6	7.2	10.8	0.0
II	Variegated Minnow, *Cyprinodon variegatus* Lacépède	4	0	95.4	0.9	1.3	2.4	0.0
	Sea Robin, *Prionotus carolinus* (Linnaeus)	4	2	96.4	0.7	1.2	1.7	0.0
	Scup, *Stenotomus chrysops* (Linnaeus)	3	2	95.3	1.4	1.1	2.2	0.0
	Butterfish, *Poronotus triacanthus* (Peck)	3	3	96.2	0.8	1.0	2.0	0.0
III	Toadfish, *Opsanus tau* (Linnaeus)	4	0	99+	rare	rare	rare	0.0
	Tautog, *Tautoga onitis* (Linnaeus)	2	3	99+	few	rare	rare	0.0
	Cunner, *Tautogolabrus adspersus* (Walbaum)	3	1	99+	few	rare	rare	0.0
	Sea Bass, *Centropristes striatus* (Linnaeus)	4	0	99+	few	rare	rare	0.0
	Pipefish, *Syngnathus fuscus* Storer	0	4	99+	few	rare	rare	0.0
	Sand Dab, *Hippoglossoides platessoides* (Fabricius)	2	0	99+	rare	rare	rare	0.0
	Weakfish, *Cynoscion regalis* (Bloch and Schneider)	0	1	99+	rare	rare	rare	0.0
	Sharksucker, *Echeneis naucrates* Linnaeus	0	1	99+	few	rare	rare	0.0
IV	Smooth Dogfish, *Mustelus canis* (Mitchill)	4	4	80.6	5.4	3.4	7.6	3.0
	Spotted Skate, *Raja diaphanes* Mitchill	1	0	83.4	4.6	3.2	6.5	2.3

The first group comprises those in which the proportion of immature erythrocytes is strikingly high, approximately 20 per cent of the total erythrocytes. In the second group the number of immature red cells is much smaller, varying from 3 to 6 per cent. In the third group, the erythrocytes were practically all mature. The fourth group includes the elasmobranchs. These are grouped separately because their erythrocyte picture is quite different from that of the teleosts, resembling more closely conditions found in the cyclostomes, Dipnoi (Jordan and Speidel, 1930, 1931), and urodeles (Dawson, 1930). All stages of maturing erythrocytes, including erythroblasts, were seen and frequently erythrocytes in mitosis were encountered (Maximow, 1923). Summarized results are shown in Table I.

In reading this table it should be borne in mind that the mature erythrocytes of different species vary in the amount of persistent reticulation, the criterion for placing them in Class I being that the reticular material exists as isolated granules or scattered, irregular filaments which may even be branched. In general, the mature cells of the fishes of Group III contain relatively more remnants of the reticulation patterns than those of Groups I and II. Probably not too much significance should be attached to the relative numbers of cells in Classes II, III and IV; the end-points for separating them into these classes are not sharp, and different observers might vary appreciably in their determination of the classes into which such cells should be placed. The figures given are at least indicative of the conditions to be expected, but for quantitative work, where the degree of maturity of the erythrocytes is of importance, reticulation estimates should be made for each species studied.

Discussion

Many of the very active fishes are included in Group I. In general these fish are characterized by their inability to survive for any extended period in restricted quarters, such as the base of the aquarium stands in the Marine Biological Laboratory, even when a moderate inflow of sea water is provided. In the case of the common mackerel this sensitivity to confinement has been found by Hall (1930) to be due, in part at least, to the peculiar adaptation of its respiratory mechanism which permits the animal to secure sufficient oxygen only when it is moving rapidly forward, the ability to respire in a stationary position having been practically lost. The same explanation may hold for some others of this group, menhaden, alewife, summer herring and bluefish, but Hall's experiments apparently have not been extended to include them. In the case of the eel, however, there is no such sensitivity to confinement; these animals may be kept in tanks for days at a time without exhibiting any signs of respiratory distress.

The fishes of Group II are less active than those of Group I and may successfully be kept in tanks, while most of those in Group III are relatively sluggish, frequently resting quietly in the water. Group IV, comprising the elasmobranchs, need not be discussed in this connection since their blood picture is quite unlike that of teleosts.

Several other sets of data compiled on the blood of teleosts parallel rather closely the present findings on the relative numbers of immature erythrocytes in the circulation. These data are based on determinations of hemoglobin concentration, size of the red corpuscles, numbers of corpuscles per cubic millimeter of blood, lowest oxygen tension at which fish are capable of removing dissolved oxygen from sea water, and the oxygen capacities of the blood.

A comparison of the hemoglobin concentration, measured in milligrams of iron per 100 cc. of blood, has been made of a group of marine fishes by Hall and Gray (1929). Highest concentrations, ranging from 41 to 35.5 mg., were found in the bonito, bull's-eye mackerel, common mackerel, and menhaden. A second group, with concentrations ranging from 23.7 to 27.7 mg., included the cunner, butterfish, scup and sea robin. The remaining fishes of this series vary more widely in this respect, showing concentrations ranging from 21.7 to 11.5 mg. Listed in a descending order these are the rudder fish, puffer, eel, silver hake, goosefish, toadfish and sand-dab. Reference to Table I will show a general agreement between my data and that of Hall and Gray, the most notable exceptions being the eel and cunner. The figures of Root (1931), covering several of the same species of fishes, also show that the high concentration of hemoglobin is associated with small cell-size, high corpuscle count, and high volume per cent of corpuscles.

It is quite evident that all those having high concentrations of hemoglobin are the more active fish. The conditions characterizing their blood presumably impose a greater burden on the erythropoietic centers in that a greater number of corpuscles must be maintained. Any considerable acceleration of the rate of erythrocyte production is rather generally accompanied by the appearance of less mature forms in the blood stream.

No satisfactory explanation has been given for this appearance of immature cells in the circulation. It is held by some that it is due to the limited volume of the erythropoietic organ. With a restricted storage capacity, partially mature cells are forced out to maintain room for the newly-forming cells (Barcroft, 1925). It is probable that some such factor as rate of erythrocyte production is operative in the fishes of Groups I and II.

The underlying stimulus for rapid erythrocyte production is not readily determined. In general the blood picture of active fishes resembles the high-altitude effect obtained in mammals, namely, an increase in total number of red cells and hemoglobin as well as an increase in the number of young cells, reticulocytes. Barcroft has pointed out that the basal principle of the effects of high altitudes is that the blood cell count is a function of the degree of anoxemia. The findings of Hall (1930) on the lowest oxygen tensions at which teleost fishes are capable of removing dissolved oxygen from sea water appear to bear directly on the problem.

Of the fishes studied by Hall the mackerel is unique, being unable to remove dissolved oxygen below a partial pressure of 70 mm. Hg. Other fishes cease respiration at the following tensions: rudderfish, 15.1 mm.; cunner, 14.8 mm.; scup, 13.1 mm.; butterfish, 11.8 mm.; sea robin, 8.5 mm.; fundulus, 8.1 mm.; puffer, 4.7 mm.; sea bass, 3.2 mm.; tautog, 1.6 mm.; and toadfish, 0.0 mm. The eleven species listed may be roughly divided into three groups. One group, represented by the mackerel, removes oxygen only at a relatively high tension, 70 mm. Hg. The second group removes dissolved oxygen between 8 and 15 mm. Hg. pressure, while the third group survives at tensions varying from 0.0 to 4.7 mm. Hg. There is thus a striking correspondence between this grouping of teleosts based on ability to remove dissolved oxygen from sea water and the grouping of the author according to the number of the immature erythrocytes present in the circulation. The cunner, however, is a notable exception.

In an earlier study by Hall (1929) of the scup and toadfish it was found that the scup showed very little change in its oxygen consumption with the oxygen tension between 120 and 40 mm. Hg. pressure but below 30 mm. Hg. pressure a very sharp decline in oxygen consumption occurred and the fishes quickly died whenever the pressure fell below 16 mm. Hg. In the case of the toadfish the oxygen consumption was found to be directly proportional to variations in the oxygen tension between 0 and 118 mm. Hg. Also the toadfish lived for 24 hours with the oxygen tension between 0 and 1 mm. Hg. pressure.

The studies of Hall (1931), Hall and Gray (1929), and Root (1931) indicate that there is a definite correlation between the transportation of oxygen and the environment and habits of the fishes. Adaptations of the respiratory mechanisms are important as shown by Hall (1930, 1931) in his studies of the puffer and mackerel, permitting one form to secure an adequate oxygen supply in a stationary position while the other can do this only when swimming rapidly forward. As already pointed out, sluggish fishes have blood with larger and less numerous corpuscles, low concentrations of hemoglobin and low oxygen capacities, while active fishes have smaller and more numerous corpuscles, higher concentrations of hemoglobin, and high oxygen capacities—definite adjustments between capacities and requirements. Further evidences of adjustment are also found in the behavior of the hemoglobin at low carbon dioxide tensions. In the presence of 1 mm. of carbon dioxide, a considerably greater tension of oxygen is required to saturate the hemoglobin of the mackerel than that of either the sea robin or toadfish. Root (1931) thinks this may account in part for the great susceptibility of the mackerel to asphyxiation (Hall, 1930).

These morphological and physiological features of the blood, representing adaptations to habits and environment, tend to make the active fish susceptible to variations in the oxygen content of sea water. The high numbers of immature erythrocytes, while probably largely the result of the normal, rapid rate of production of these elements, may also tend to be further increased by stimuli resulting from anoxemia. In other words the margin of safety of active fishes is reduced and the normal condition of their blood may readily pass into a state characteristic of the high-altitude effect found in mammals.

These observations immediately raise the question as to what extent the proportions of immature cells found in the teleosts studied are due to the immediate response of these fishes to varying degrees of asphyxiation caused by methods of transportation and retention in tanks. Studies on the effects of asphyxiation on the menhaden and puffer have been made by Hall, Gray and Lepkovsky (1926) and Hall (1928). These observations showed that asphyxiation produced a definite increase in the concentration of hemoglobin and iron and in the number of red-blood corpuscles, the increase being roughly proportional to the length of time of asphyxiation (19 to 50 minutes). The increased concentration of the blood constituents was explained by a release of water from the blood into the tissues, since the blood volume became diminished during asphyxiation. Iron, however, did not increase in proportion to the loss of water or to the increase in concentration of hemoglobin, but at a faster rate. The spleen showed a decrease in size during asphyxiation and it was suggested that the spleen is a store-house for iron which is released into the blood during asphyxiation.

The observation on the reduction in the size of the spleen may be significant, since in the teleosts this constitutes the major erythropoietic center. In the menhaden, the red-blood cell count rose from 1,988,000 to 3,598,000 per cubic millimeter during 50 minutes confinement in a sealed jar containing 10 liters of water. Since differential counts for degree of persistent reticulation were not made, it is impossible to determine whether the increased number of corpuscles per cubic millimeter was due solely to the withdrawal of water or whether additional and probably less mature erythrocytes had left the spleen to enter the circulation. If erythrocytes do leave the spleen during asphyxiation, as the decrease in its size suggests, it would seem to constitute a possible source of the immature erythrocytes encountered in the circulation.

In the present study the environment of the fishes, especially as regards oxygen tension, was not rigidly controlled. Fish were brought from the traps in the morning and delivered directly to the laboratory in pails of sea water, and many of the more active forms such as mackerel, menhaden, alewife, and summer herring frequently exhibited obvious signs of respiratory distress when received. However, counts made from alewife and mackerel taken directly from a floating " live car " did not differ appreciably from those made on fish delivered by the collecting staff to the laboratory. Since the oxygen tension of the sea water was not determined at the time blood was removed, no statement can be made as to the degree of asphyxiation, if any, to which the fish were subjected.

The prime purpose of this survey was to ascertain the relative homogeneity of the erythrocyte picture in fishes under ordinary laboratory procedures when no particular precautions are taken to insure an optimum environment—conditions such as may prevail when blood is obtained for studies on hemolysis and erythrocyte respiration. If the varying degrees of asphyxiation to which the fish were possibly subjected have modified the blood picture as described in this paper, then such errors are inherent in the data and no correction for them can be made at this time.

Accordingly the variations in the numbers of immature cells either may represent the varying ability of the fishes to respond to the adverse conditions encountered following removal from their natural environment or are evidences of the specific adjustments that have been made between the production of the oxygen-transporting elements, the red-blood cells, and the structural adaptations, the environment, and the habits of the fishes. In fact, these alternatives need not be mutually exclusive. The response to asphyxiation, if proven to occur, may be but an exaggerated picture of conditions normally present in a particular species.

In the elasmobranchs, as in the cyclostomes, Dipnoi, and urodeles, differentiation and multiplication of erythrocytes takes place to a variable extent in the peripheral circulation, and all stages, from their hemoblast progenitors to the completely differentiated cells, may be encountered. Mitosis may also occur in all stages of differentiation, short of actual maturity. The counts include only cells which contain hemoglobin, the hemoblasts and proerythroblasts being omitted, and are based on large, freshly-caught specimens.

Young dogfish which had been kept in "live cars" for some time showed a higher number of immature cells as well as an increase in the number of mitoses. The blood appeared to be in an actively regenerative phase, probably brought about by the conditions of confinement and diminished food supply. The blood picture of elasmobranchs stands in striking contrast to that of teleosts where the erythrocytes enter the circulation in a much later stage of differentiation and no proliferation by mitosis is observed in the peripheral circulation.

SUMMARY

The results of a study of the blood of the general circulation of twenty species of marine fishes are given. The number of immature erythrocytes present varies widely.

The differential erythrocyte counts were based largely on supravital preparations stained with brilliant cresyl blue. This material was supplemented by dry-fixed smears stained by Wright's method. The reticulation patterns produced by brilliant cresyl blue are discrete structures and more accurate counts can be made on these preparations than on stained smears where the varying degrees of basophilia and polychromasia are used as criteria of immaturity.

The twenty species of fish examined fall naturally into four groups. In Group I, including the mackerel, menhaden, alewife, summer herring, bluefish and common eel, the proportion of immature erythrocytes is high, approximately 20 per cent. In the second group, including the variegated minnow, sea robin, scup, and butterfish the percentage of immature cells is lower, varying between 3 and 6 per cent. In the third group, including toadfish, tautog, cunner, sea bass, pipefish, sand dab, weakfish and sharksucker, practically all the erythrocytes are mature. The fourth group, comprised of elasmobranchs, was treated separately since in these forms erythrocytes are continuously differentiated in the blood stream from primitive cells and may also proliferate mitotically in this location. The number of immature red cells in these fish is also great.

The varying blood pictures appear to represent the result of specific adaptations of fishes to such interrelated factors as their type of external respiratory mechanism, the efficiency of their oxygen-transporting system, their oxygen requirements and the oxygen tensions of their environment. In Group I the general blood picture is suggestive of the high-altitude effect observed in mammals.

Many of the fishes with high counts of immature erythrocytes quickly exhibit signs of asphyxiation when removed from their natural habitat and kept in tanks. Hall has noted that during asphyxiation the spleen of fishes decreases in volume. This may be correlated with the entrance of immature erythrocytes into the general circulation. Accordingly, it is possible that the figures presented in this paper are not exactly representative of the condition of the blood when such fish are undisturbed in their natural environment.

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ON THE RESPIRATORY FUNCTION OF THE BLOOD OF THE PORPOISE

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THE WOODS HOLE OCEANOGRAPHIC INSTITUTION [1]

The capture of two porpoises, *Phocæna phocæna* Linn., in the fish trap operated by the Marine Biological Laboratory has afforded an opportunity to obtain data which have not been available before on the conditions of equilibrium between the blood of a cetacean and the respiratory gases. The mammals of this order are so strikingly modified morphologically in adaptation to their aquatic life that an examination of the physico-chemical properties of the respiratory fluids is attended with some interest. While the results recorded in the present paper require confirmation and extension in many details, the general picture of the physico-chemical system involved in transporting the respiratory gases is sufficiently definite to warrant presentation in the present provisional form.

The porpoises were brought from the trap on May 14 and placed in a large floating crate at the laboratory. One died on the following day and was used to determine the proper approach for cardiac puncture. The other lived until May 18, when it was killed in the course of experiment. During this period it lay in the water rather quietly and did not struggle when held. On May 17 fifty cubic centimeters of blood was drawn by puncture of the left ventricle, the needle being inserted one inch behind and above the caudal edge of the left flipper. The porpoise did not struggle. A 10 cc. portion of this blood was preserved under oil, for the determination of the carbon dioxide content of arterial blood. The remainder was used for the determination of the shape of the oxygen and carbon dioxide dissociation curves. Coagulation was prevented by the use of dry sodium oxalate. The blood drawn at this time will be designated *Specimen A*.

The following day an unsuccessful attempt was made to measure the blood volume by the vital red method. Twenty cubic centimeters of blood were drawn from the heart with some difficulty and it appeared that both right and left ventricles had been pierced as a portion of the sample was not fully oxygenated. This sample was oxalated and used for the determination of oxygen capacity and cell volume and was designated *Specimen B*.

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[1] Contribution No. 4.

Immediately after drawing Specimen *B*, the porpoise went into asphyxial convulsions, evidently as the result of cardiac failure. The thorax was opened and 50 cc. of blood were drawn directly from the ventricle. This sample was designated as *Specimen C* and was used for determining the effect of hydrogen ion concentration upon the position of the oxygen dissociation curve.

METHODS

Oxygen and carbon dioxide contents were determined by using the Van Slyke constant volume analyzer. The gas mixtures were analyzed with a Haldane apparatus after equilibration and the pressures calculated by means of the equation developed by Bock, Field, and Adair (see Henderson, 1928, p. 384). The blood was equilibrated with the gas mixtures at 38°, preserved in blood-sampling tubes over mercury, and promptly analyzed. The original specimens of blood were preserved in the refrigerator until used, and all measurements were made within twenty-four hours of the time the specimens were secured.

TABLE I

Oxygen Capacity and Volume of Erythrocytes in the Blood of Aquatic Mammals

Species	Porpoise Phocæna phocæna			Dolphin Tursiops tursio	Sperm Whale Physeter macrocephalus	Sea Lion Eumetopias stelleri
Specimen	A	B	C			
Oxygen capacity, vol. per cent	22.15 22.20	19.8 19.7	20.44 20.34			19.8
Oxygen combined, vol. per cent	21.78	19.35	19.99	31.8	29.09	19.4
Volume of cells in 1 cc. blood, cc.	0.35	0.35	0.36	0.517		0.29
Oxygen combined in 100 cc. cells, vol. per cent	62.2	55.4	55.6	61.5		67

THE OXYGEN CAPACITY OF THE BLOOD AND OF THE ERYTHROCYTES

Table I contains the data obtained from the three specimens of blood we have examined, together with results reported by Jolyet (1902) for the dolphin and sperm whale, and by Florkin and Redfield (1931) for the sea lion. The oxygen capacities we obtained, like those observed in the sea lion, are of the same magnitude as those commonly found in terrestrial mammals. They contrast strongly with the higher values recorded by Jolyet and Sellier (1896–97) for the porpoise (30.9 volumes per cent), by Jolyet (1902) for the dolphin and sperm whale, and with the values of 42.5 and 45.1 volumes per cent reported by Sudzuki (1924) for the blood of porpoises. Whether these differences are characteristic of the different species in question, or are due to accidental causes, such as a possible anæmic condition of the individuals yielding the lower values, or to experimental errors, cannot be decided until more data are available. We were impressed with the rapidity with which the corpuscles settled out when the porpoise blood was allowed to stand and believe that errors large enough to account for the differences reported for the different species might readily arise from this cause were it not guarded against. The observations are all in agreement in showing that a given volume of the corpuscles of these aquatic mammals combines more oxygen than does an equal volume of corpuscles of the terrestrial mammals, in which 100 cc. of corpuscles have an oxygen capacity of about 45 cc.

TABLE II

Data on carbon dioxide equilibrium in porpoise blood. Temperature, 38° C.

	Oxygen pressure	Carbon dioxide pressure	Carbon dioxide content
	mm. Hg	*mm. Hg*	*vol. per cent*
Specimen A	150 ca.	30.7	35.8
	150 ca.	51.3	46.0 46.2
Specimen C	150 ca.	41.0	25.6 25.5
	150 ca.	51.1	29.7 29.0
	1.4	51.8	32.45 32.30

THE CARBON DIOXIDE DISSOCIATION CURVE

Table II records the data obtained by equilibrating Specimens *A* and *C* with carbon dioxide. From these data the carbon dioxide dissociation curves illustrated in Fig. 1 have been constructed by making use of the approximation (Henderson, Bock, Dill, and Edwards, 1930):

$$\log T = A \log (p\text{CO}_2) + B,$$

in which T is the total carbon dioxide content of the blood, A and B are constants, and $p\mathrm{CO}_2$ is the carbon dioxide pressure. The curve constructed from data on Specimen *A* does not differ greatly from that of normal man, though the carbon dioxide combined at any pressure is somewhat less. The curve for Specimen *C* represents blood drawn following death from asphyxia, and the combined carbon dioxide is consequently less at each carbon dioxide pressure.

These curves may be used for comparing the buffer action of porpoise blood with that of human blood. Henderson, Bock, Dill, and Edwards (1930) have prepared a nomogram showing the relation between the slope of the carbon dioxide dissociation curve, its height, and the hemoglobin content of human blood based on an examination of some 117 specimens. In Table III are recorded the values of *Hb,* the hemoglobin content as measured by oxygen capacity; T_{40}, the carbon dioxide content at 40 mm. carbon dioxide pressure; and Δ, the difference in carbon dioxide content at 30 and 60 mm. carbon dioxide pressure as deduced from the curves and data describing Specimens *A* and *C*. In this table are also recorded the values of Δ characteristic of human blood having equivalent values for *Hb* and T_{40}, taken from the nomogram of these authors. Both specimens of porpoise blood have slightly higher values of Δ than does comparable human blood. The differences, however, are not greater than might be accounted for by experimental error. Comparable data for the blood of the sea lion, from the measurements of Florkin and Redfield (1931), and mean

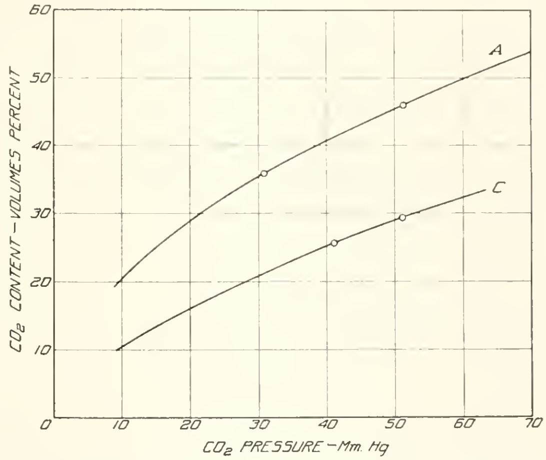

FIG. 1. Carbon dioxide curve of blood of porpoise. Upper curve, Specimen *A*. Lower curve, Specimen *C*. For data see Table II.

values for dog blood given by Dill, Edwards, Florkin, and Campbell (1932) are included in the table. The comparison serves to emphasize the similarity of the bloods when regarded as systems for transporting carbon dioxide.

Table II contains measurements of the carbon dioxide content of a sample of Specimen *C* equilibrated with carbon dioxide in the virtual absence of oxygen. Comparing the results with the value obtained from the curve in Fig. 1 for the same carbon dioxide pressure, it appears that the reduced blood combines about 2.86 volumes per cent more carbon dioxide than does oxygenated blood. This effect is similar to that observed in other mammalian bloods but is rather smaller than usual for blood of comparable oxygen capacity.

TABLE III

Comparison of Buffer Value of Oxygenated Porpoise and Sea Lion Blood with that of Man

Species	*Hb* Oxygen capacity	T_{40} CO_2 content at $pCO_2 = 40$ mm.	Δ observed	$T_{60} - T_{30}$ calculated from human blood	Ratio
	milliequiv. per liter	*milliequiv. per liter*	*milliequiv. per liter*	*milliequiv. per liter*	
Porpoise					
Specimen A	9.73	18.18	6.52	5.80	1.12
Specimen C	8.93	11.22	5.12	4.70	1.09
Sea Lion	8.88	16.02	5.32	5.30	1.00
Dog (Mean)	9.7	18.1	5.78	5.76	1.00

THE OXYGEN EQUILIBRIUM

Data on the equilibrium of Specimen *A* with oxygen are presented in Table IV. In order to fit the measurements, the oxygen dissociation curve shown in Fig. 2 was drawn using Hill's equation

$$y = \frac{Kx^n}{1 + Kx^n} \times 100,$$

in which y is the percentage saturation of the hemoglobin, x the oxygen pressure, $K = 0.00102$ and $n = 2.1$. The carbon dioxide pressure of these samples was about 46.5, corresponding to a carbon dioxide content of 44 volumes per cent. Taking $pK = 6.14$, yields a value for pH of 7.29. The values for n which have been used to characterize the blood of man vary from 2.2 to 2.5. The shape of the oxygen dissociation curve of the porpoise so far as it is characterized by n does not differ markedly from human blood.

The portion of Specimen *A* which was collected under oil had a carbon dioxide content of 45 volumes per cent, corresponding to a carbon dioxide pressure of 49 mm. and a pH of 7.27. Consequently the oxygen dissociation curve in Fig. 2 represents approximately the conditions characteristic of the blood *in vivo,* if one neglects the change in oxygen equilibrium, due to the increased carbon dioxide content of the venous blood.

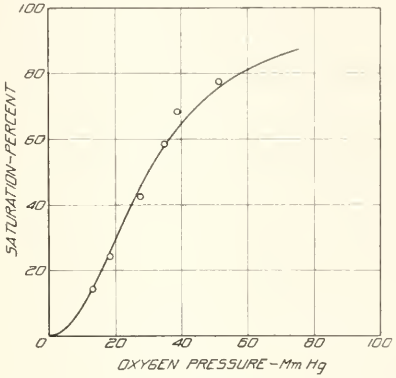

FIG. 2. Oxygen dissociation curve of blood of porpoise at a carbon dioxide pressure of approximately 46 mm.—equivalent to that of arterial blood. For data see Table IV.

THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE OXYGEN DISSOCIATION CURVE

Table V contains data for the oxygen equilibrium of samples of Specimen *C* obtained at a variety of carbon dioxide pressures. Using

TABLE IV

Data on the equilibrium of oxygen with porpoise blood, Specimen A.
Temperature, 38° C.

Carbon dioxide pressure	Oxygen pressure	Oxygen content	Oxygen dissolved	Oxygen as oxyhemoglobin	Saturation
mm. Hg	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*
46.5	13.0	3.14	0.03	3.11	14.3
46.4	18.1	5.30	0.05	5.25	24.1
46.6	27.5	9.37	0.07	9.28	42.5
47.2	34.9	12.86	0.09	12.77	58.6
38.7	38.7	14.95	0.12	14.83	68.1
42.7	51.4	16.98	0.15	16.83	77.3
air	air	22.18	0.40	21.78	100.0

Hill's equation and taking the value of $n = 2.1$, the values of K, and of p_{50} (the oxygen tension at which the blood would be half saturated with oxygen) have been calculated. As an empirical procedure this practice involves only the assumption that the form of the oxygen dissociation curve, determined by the value of n, remains unchanged at various carbon dioxide tensions. This assumption is amply justified by the behavior of other mammalian hemoglobins. The hydrogen ion concentration of the various samples have also been calculated with the aid of the carbon dioxide dissociation curve for Specimen C in Fig. 1. From these values the relation between p_{50} and pH shown in Fig. 3 has been plotted.

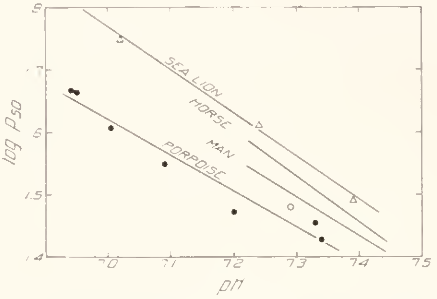

FIG. 3. Relation of p_{50}, the oxygen tension at which blood is half saturated, and the pH of the serum of whole blood. For data see Table V and references in text. Hollow circle, porpoise Specimen A. Solid circles, porpoise Specimen C.

For precise comparison of the properties of hemoglobin in the blood of different animals the properties should be referred to the pH of the cell contents. Unfortunately our studies did not include the measurements necessary to enable us to calculate this value. We have conse-

TABLE V

Data on the equilibrium of oxygen with porpoise blood (Specimen C) at different carbon dioxide tensions. Temperature, 38° C.

Carbon dioxide pressure	Oxygen pressure	Saturation	log p_{50} calc.	pH
mm. Hg	*mm. Hg*	*per cent*		
9.86	34.4	62.7	1.428	7.34
10.5	23.5	40.0	1.455	7.33
21.4	18.4	26.7	1.473	7.20
39.7	27.1	36.3	1.550	7.09
52.5	45.1	56.5	1.607	7.04
87.6	38.9	41.2	1.664	6.95
92.0	54.4	58.3	1.666	6.94

quently compared the values of p_{50} obtained at different carbon dioxide pressures with those characterizing other bloods when the pH of the serum of the whole blood was the same. This comparison is included in Fig. 3, the data for the horse and for man being taken from the nomograms given by Henderson (1928); that for the sea lion is calculated from the data of Florkin and Redfield (1931). When compared on this basis the hemoglobin of the porpoise does not differ strikingly from that of other mammals and does not differ from the blood of normal man much more than the blood of men differ from one another.

Discussion

The preceding observations all tend to indicate that the general physico-chemical properties of the blood of the porpoise are very similar to those of the terrestrial mammals. Even in the strictly quantitative aspects of the various properties of the blood one can detect very little variation from the mean conditions observed in human blood. The increased concentration of hemoglobin in the corpuscles of the cetaceans and the sea lion is perhaps the only clear-cut condition which might be regarded as an adaptation to the more rigorous respiratory requirements of aquatic life.

Unfortunately, our attempts to determine the blood volume of the porpoise were unsuccessful. This relation is, in our opinion, the most important aspect of the respiratory mechanism which remains to be examined. One cannot help being struck by the richness of the muscular tissue in hemoglobin. It seems probable that in this condition, and perhaps in other modifications of the chemical situation in the muscles, adaptations to aquatic life may be found.

It should be pointed out that the porpoise is not in the habit of remaining submerged for long periods of time when compared to the larger Cetacea and certain other aquatic mammals. Parker (1932) records that in a porpoise confined in a large tank, the average respiratory interval was 15.48 seconds. The shortest interval observed was 6.5 seconds, the longest 31.7 seconds. These are not strikingly greater than the intervals of inspiration in man and might easily be maintained, at least temporarily, by voluntary effort in his case. When feeding in the open sea, one might expect the intervals to be greater and Jolyet and Kükenthal (quoted by Parker) note that dolphins may remain under water much longer, Jolyet setting the maximum at about fifteen minutes. The general testimony of whalers indicates that the larger Cetacea are in the habit of remaining submerged for much longer periods. Whether more distinct adaptations of the physico-chemical mechanisms of the blood will be found in such forms remains to be seen.

Summary

The properties of the blood of the porpoise, *Phocœna phocœna*, considered as a system for the transport of oxygen and carbon dioxide, have been examined.

It does not appear that the morphological changes adapting these animals for aquatic life are accompanied by significant modifications in the physico-chemical properties of the blood.

The only striking characteristic of porpoise blood, when compared with that of terrestrial mammals, is an increased concentration of hemoglobin in the corpuscles.

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THE EXTERNAL FIBRILLAR SYSTEM OF EUPLOTES WITH NOTES ON THE NEUROMOTOR APPARATUS

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The nature of the external fibrillar system of ciliate Protozoa (" Silberliniensystem " of Klein, 1926*a*, 1926*b*) and its relation to the better-known neuromotor apparatus as first described by Sharp (1914) for *Diplodinium* has been the subject of considerable research of late years. Marked differences of opinion obtain as to the function of both these types of fibers and even their structure has not been agreed upon.

With the hope of solving some of the problems concerned, *Euplotes patella* was chosen for the subject of this investigation chiefly because its neuromotor system is probably better known than that of any other ciliate. Its description given by Yocum (1918) and the demonstration of its function by Taylor (1920) serve as two of the chief milestones toward an understanding of the fibrillar makeup of ciliates.

The description of the external fibrillar network in *E. patella* was originally undertaken by Mr. Samuel Yabroff in 1925 at the University of California. Later he gave up this work when he entered medical school and turned the problem over to me with the statement that he was no longer interested in it.

This work was aided by a research grant from the Graduate School Research Fund of the University of Minnesota.

TECHNIQUE

Euplotes for this study was cultivated in mass on wheat and timothy hay as described by Turner (1930). The methods of fixation and staining or silver impregnation are outlined below.

For the External Fibrillar System

Silver method of Klein (1926*b*). This gave fair results but drying caused too much distortion. Fixing first in osmic vapor helped some.

Brown's (1930) method of using thionin after Mann's fixation did not show the fibrils at all. Like Liebermann's (1929) nigrosin method it is excellent for *Paramecium* but useless for *Euplotes*.

Silver-gelatin method of Chatton and Lwoff (1930). A few specimens that are around the border of the main drop of gelatin show the network very well, but it is difficult to get just the proper amount of gelatin on the slide.

Formol-osmium-toluidin blue method of Gelei (1927) was useless as no differentiation could be obtained.

The technique which consistently gave the best results for whole preparations was a variation of Klein's and Gelei and Horváth's (1931) methods: Place a small drop of material containing the organisms on a clean slide previously smeared with a light coat of egg albumen. Draw off excess water with a micro-pipette. Fix with osmic acid vapor for about three seconds. This may be easily done by simply inverting the slide over a bottle containing 2 per cent osmic acid. Place slide in a cool breeze (4° to 15° C.) until the material is nearly but not quite dry in the center. Flood gently with two or three drops of 2 per cent silver nitrate. A few of the organisms will be lost but most of them will be caught in the albumen. After 4 to 8 minutes in the silver nitrate, pour off the excess and place the slide in a white dish, or glass dish over white paper, containing enough distilled water to cover the slide. Place in the sun in a cool place and watch the progress of the reduction by occasional examinations with the microscope. When the desired depth has been obtained, wash thoroughly in distilled water, dehydrate, and mount. If slower reduction is desired, exposure to bright sky but not to the sun is efficacious. This method gives strikingly clear-cut results.

Aside from the silver impregnation methods the only way found of demonstrating the network with any degree of clarity and completeness is by drying specimens on a clean slide and staining with thionin. This is done by simply flooding the specimens with a 0.5 per cent aqueous solution of the stain for a few seconds, rinse and dehydrate as rapidly as possible, clear and mount. While this method gives essentially the same picture as the silver methods, less contrast is obtained by it.

Bresslau's (1921) method was used but did not prove satisfactory for *E. patella* although it is good for *Paramecium*.

For sectioning, the only method worth mentioning favorably out of the many tried is that of Gelei and Horváth (1931), using the maximum strength and time for the silver nitrate. After clearing, some of the material was mounted whole and the rest embedded and sectioned 3 to 15 microns thick. These sections were valuable chiefly in showing the location of the fibers in the protoplasm.

Although I have tried many times to repeat the method used by Yabroff (1928) on *Euplotes*, I have consistently failed to get satisfactory results. Others in private correspondence have reported similar failures, but Pickard (1927) reports conspicuous success with it on *Boveria*. In my opinion it is a capricious technique but one which has possibilities and with good fortune and the proper twist of the wrist will be found valuable. *Intra vitam* stains used were neutral red, Janus green B, dahlia violet, methylene blue, dilute hæmatoxylin, and thionin. All were tried in concentrations of from 1–1,000 to 1–100,000. Neutral red at about 1–10,000 gave the best results.

For the Neuromotor Apparatus

Zenker's and the picro-mercuric fluid of Yocum, followed by Mallory's connective tissue stain as used by Sharp. Sections 2, 4, 6, 8, 10, 15, and 20 microns thick were made in addition to whole mounts. Beautiful preparations were obtained with this stain following either fixative, but the colors fade. In the thicker sections and the whole mounts the general picture is seen fairly well, while the details may be better studied in the thinner sections. Whole mounts were usually fixed in picro-mercuric as the high alcohol-ether content caused the organisms to adhere to the slides much more readily than did Zenker's.

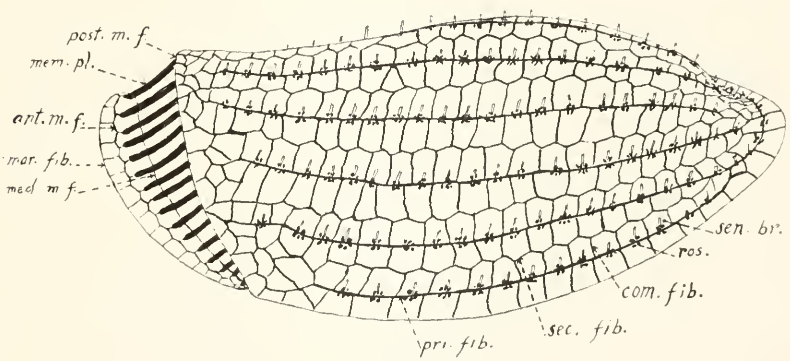


FIG. 1. Dorso-lateral view of external fibrillar system of *Euplotes patella*. Rosettes and sensory bristles from neutral red specimen, the rest from silvered specimen. *Ant. m. f.*, anterior membranelle fibril; *com. fib.*, commissural fibril; *mar. fib.*, marginal fibril: *med. m. f.*, median membranelle fibril; *mem. pl.*, membranelle plate; *post. m. f.*, posterior membranelle fibril; *pri. fib.*, primary fibril; *ros.*, rosette; *sec. fib.*, secondary fibril; *sen. br.*, sensory bristle.

Delafield's, Heidenhain's, Apáthy's and Dobel's hæmatoxylins were used following Schaudinn's, both hot and cold, and Flemming's strong fixing fluids. Schaudinn's followed by Heidenhain's gave the best results with whole mounts while Flemming's and Heidenhain's proved the most satisfactory for sections.

Many other methods were tried without conspicuous success.

Intra vitam stains as mentioned above for the external fibers were used. Neutral red 1–20,000 allowed to act for 1 to 3 hours, and thionin of about the same dilution were found most useful.

THE EXTERNAL FIBRILLAR NETWORK

The convex dorsal surface of *Euplotes patella* shows seven to nine longitudinal rows of granules arranged in little rosettes (Fig. 1). From the center of each rosette a bristle protrudes externally. Griffin (1910) states that in *E. worcesteri* sensory bristles protrude from these rosettes, and recently Jacobson (1931), using a silver technique, describes and figures a central bristle protruding from a ring instead of a rosette, in *E. patella*.

I have observed these bristles not only in material impregnated with silver, but also in living specimens stained *intra vitam* with neutral red. Each bristle is about two microns long and perhaps one-tenth micron thick. Its base is surrounded by four to eight elongate granules arranged radially (Fig. 2). In silver preparations the granules of the rosette are usually clumped into a single blob as seen in the photomicrograph (Pls. I, II, and III). That the bristles are not cilia is evident from the fact that they are not vibratile, although it is quite possible they have been evolved from ancestral cilia.

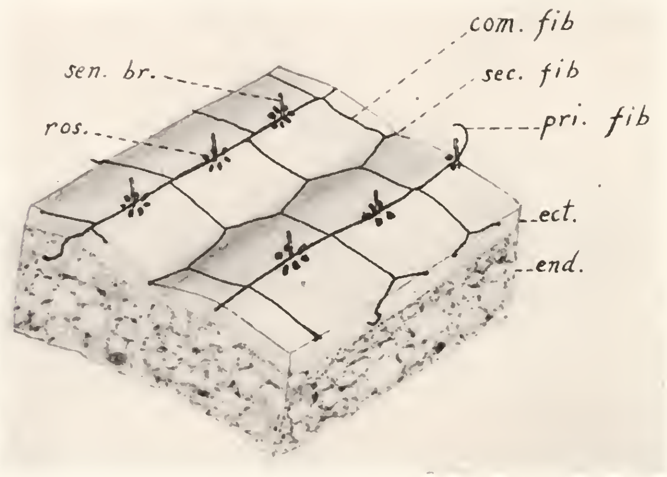


FIG. 2. Enlarged section of dorsal body wall to show position of fibrils in ectoplasm. Note several places where fibrils were not cut off clean with the section. *Ect.*, ectoplasm; *end.*, endoplasm; other labels as in Figure 1.

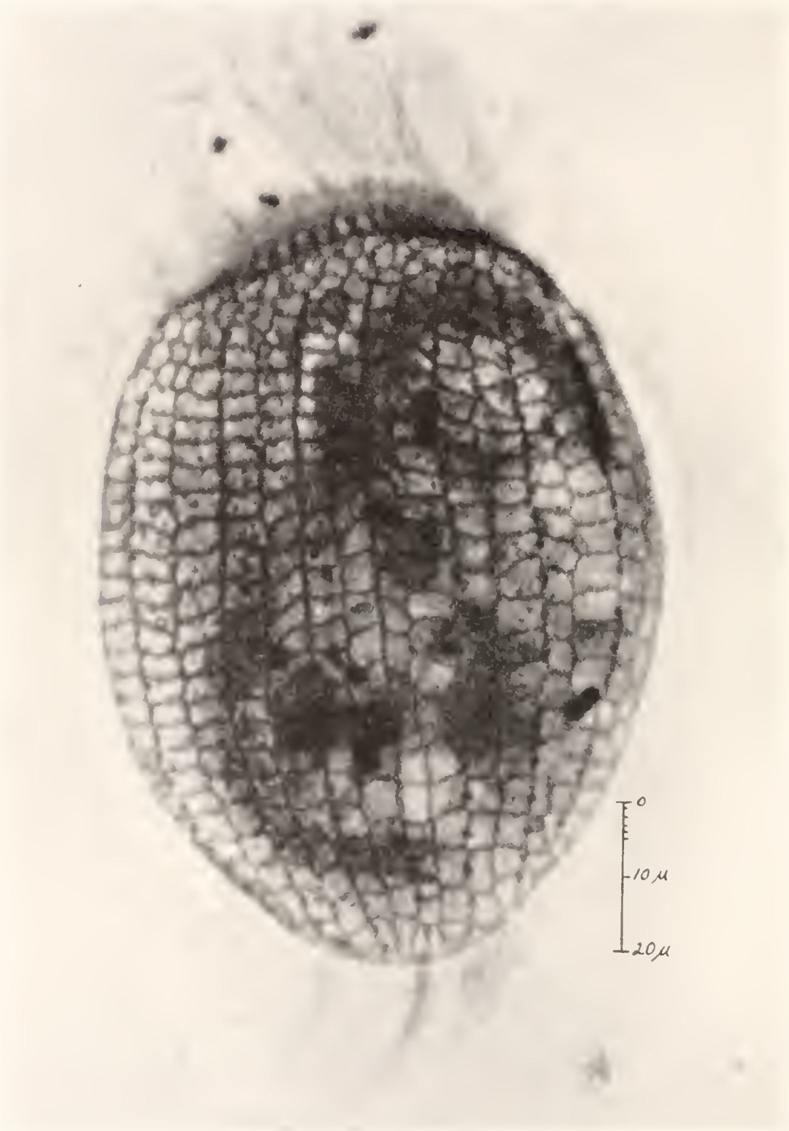
Connecting up the rosettes are seven to nine (usually nine) longitudinal fibrils. These I have called the *primary fibrils* as they are the heaviest and most easily seen, and because they are associated with the bristles and rosettes. Halfway between the primaries and extending parallel to them are the *secondary fibrils* which are only slightly less regular than the primaries. In addition to these there are *commissural fibrils* extending across from the primaries to the secondaries causing the secondaries to appear as though pulled slightly out of line. This creates a veritable network or latticework which is remarkably constant in appearance, the squares of which average about four microns across (Pls. I and II). All these structures have been observed in neutral red material demobilized with osmic acid vapor. This reminds one of Fig. *F* in Pickard's (1927) paper on *Boveria*. Myonemes, however, have no place in *Euplotes* as the cortex is rigid.

Anterially, the network is connected with the *posterior membranelle fiber* (Fig. 1) at the edge of the dorsal cortex. Both primaries and secondaries are fastened to this fibril either directly or by a more or less irregular anastomosis of the fibrils.

Anterior to the dorsal cuirass is the collar in whose surface is seen an extension of the fibrillar system, part of which was described by Yocum. Extending anterio-ventrally is the row of basal plates of the membranelles (Fig. 1). These are attached to the *posterior membranelle fibril* at the proximal end and extend about two-thirds the distance to the margin of the lip where they connect with the *anterior membranelle fibril* by means of short commissures that extend from their tips. This anterior membranelle fibril is the " membranelle fiber " (anterior cytostomal fiber) of Yocum's neuromotor apparatus. This in turn is connected to the marginal fibril by short commissures. Linking up the basal plates of the membranelles is still another fibril about two-thirds the distance to their anterior tips. This *median membranelle fibril* is seen only in the clearest preparations as it is easily blocked out by the heavy impregnation of the membranelle plates. In all probability it was part of this collar equipment that Yocum saw and considered sensory structures.

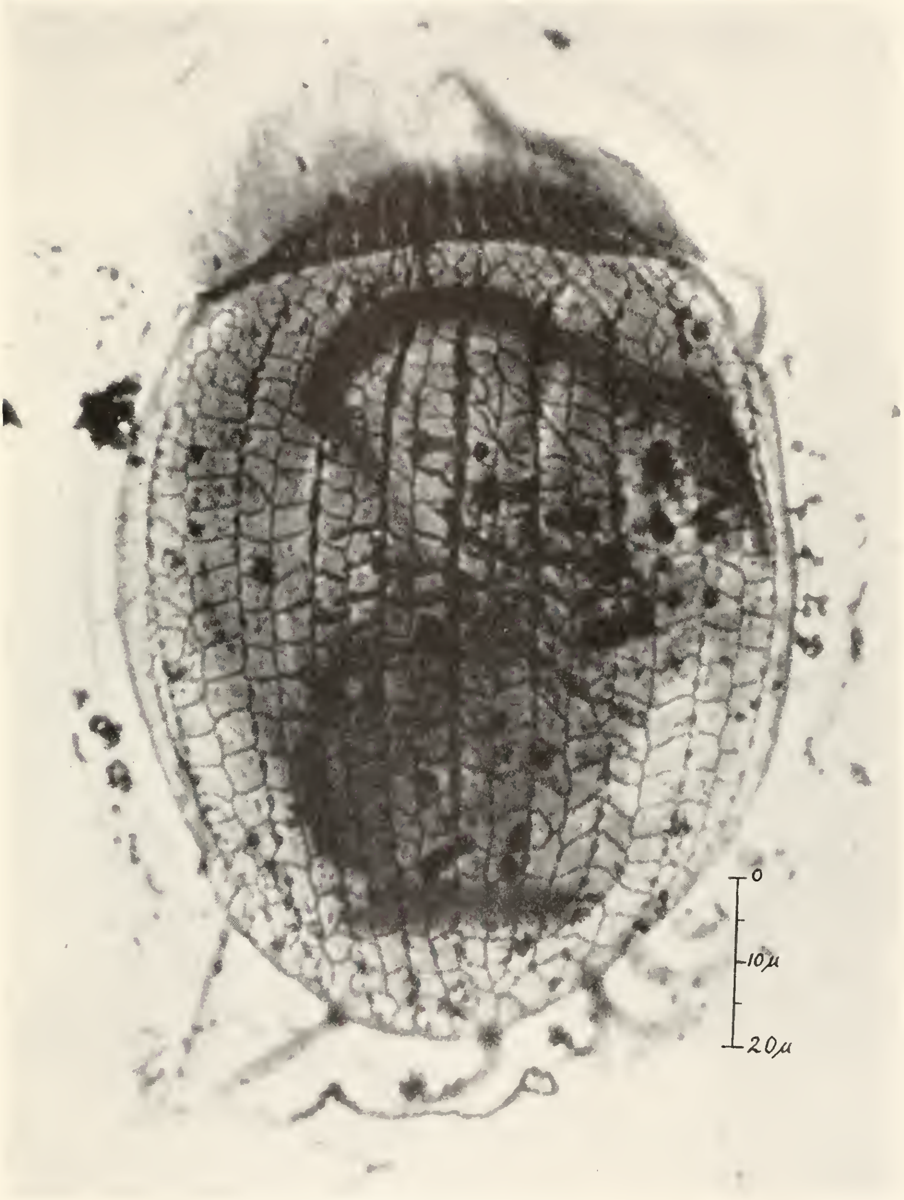
On the ventral surface of *E. patella* the network, instead of being composed of a cross-hatching of lines, appears less regular and reminds one of badly treated chicken wire (Plate III). Hexagons form a rather prominent part of the network while rectangles, squares, etc., are not infrequent. The pattern, however, as seen in any of the individuals is surprisingly constant and characteristic. For example, the hexagons of the ventral surface of the oral lip, the long slender rectangles extending posterior to the tip of the peristomal field, and the squares of the lateral phlanges are always present. The squares of the lateral phlanges are made by fibrils similar to those of the dorsal surface, the marginal fiber being a secondary and the submarginal being a primary fibril provided with rosettes and sensory bristles as are those of the dorsal surface.

LIBRARY



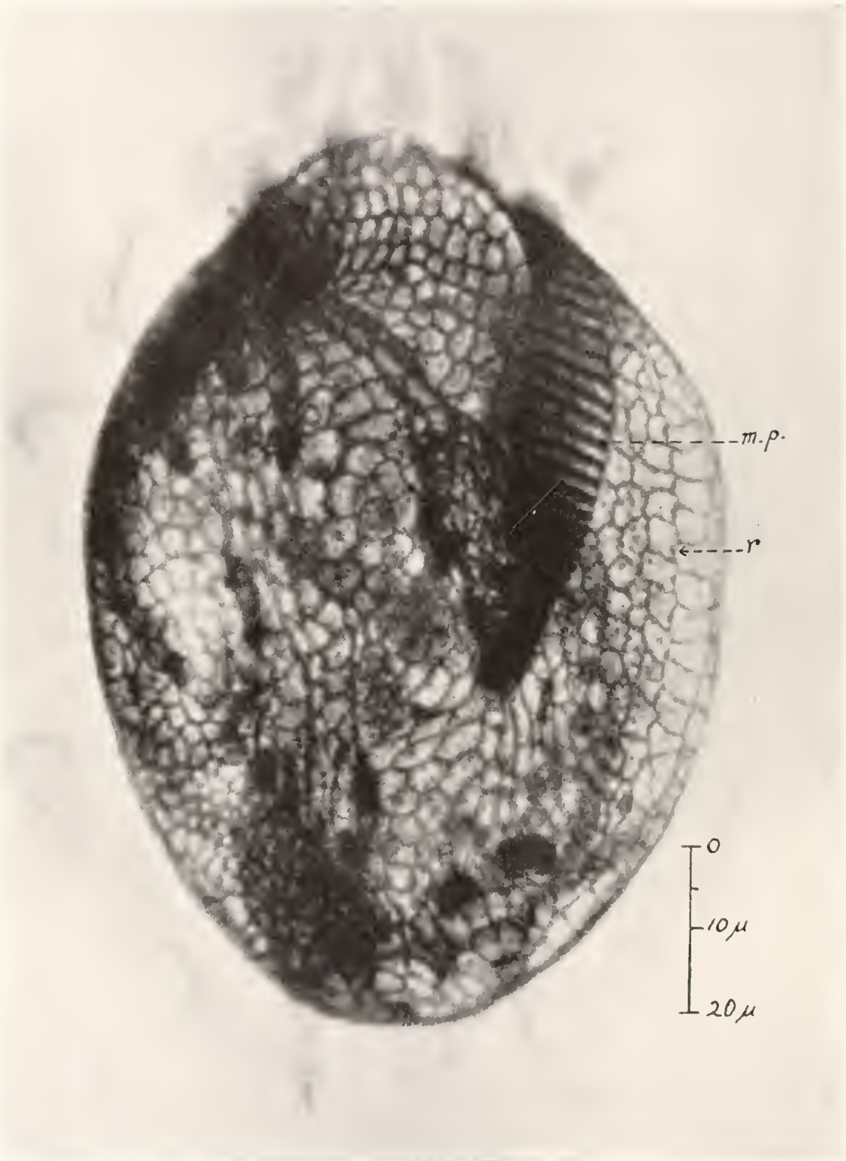
Explanation of Plate I

Photomicrograph of dorsal network of *E. patella*. Silver nitrate preparation.



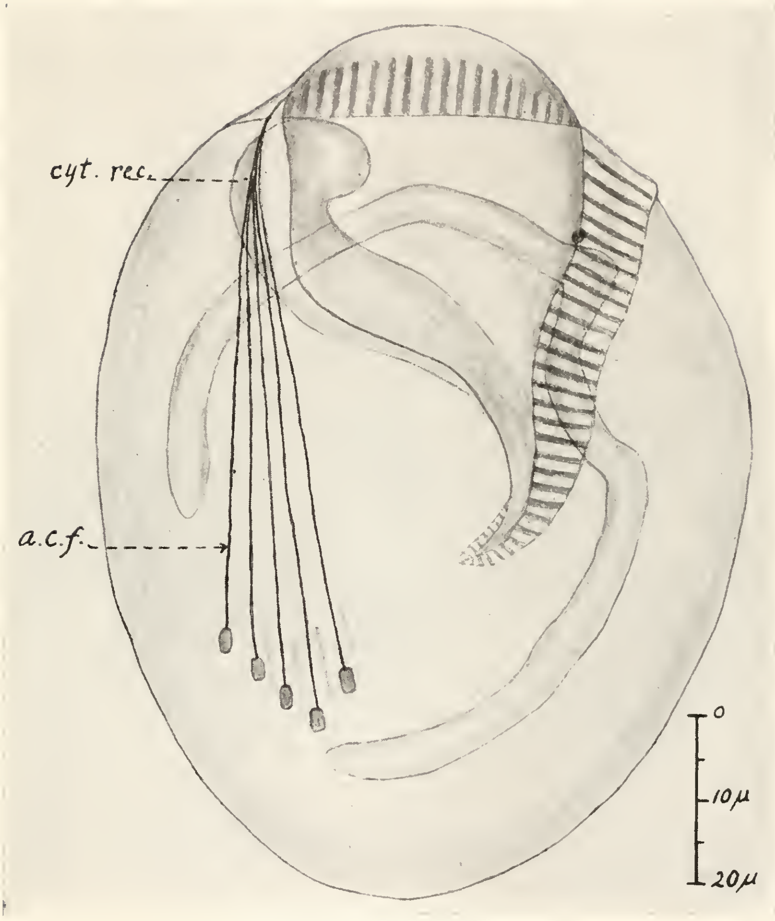
Explanation of Plate II

Photomicrograph of dorsal network of another specimen. Rosettes can be seen clearly near the sides as single blobs. Silver nitrate preparation.



Explanation of Plate III

Photomicrograph of ventral network of *E. patella*. *m. p.*, membranelle plate in lateral peristomal field; *r.*, rosette. Silver nitrate preparation.



Explanation of Plate IV

Camera lucida drawing, ventral view, of *E. patella* showing the anal cirri fibers of the neuromotor apparatus passing directly into the collar, unbroken by a motorium. All other cirri and fibers omitted from the drawing. *A. c. f.*, anal cirri fibers; *cyt. rec.*, cytostomal recess. Schaudinn's and Heidenhain's hæmatoxylin.

The basal plates of the membranelles located in the left margin of the peristome (Plate III and Fig. 3) are deeply impregnated by the silver and appear like ties of a railroad track. Extending down the center of the "roadbed" like a loosely strung wire is the fibril which connects basal plate to basal plate—a continuation of the median membranelle fibril. Bordering each side of the peristomal field of membranelles and in direct contact with the ends of the basal plates are the two other membranelle fibrils.

Sectioned material shows the fibrils of both dorsal and ventral networks to be immediately under the pellicle and in contact with it. They appear round in cross-section and the fibrous nature is disclosed by the fact that frequently they are not cut off clean with the section but protrude from the edge like loose threads at the end of a frayed piece of cloth (Fig. 2).

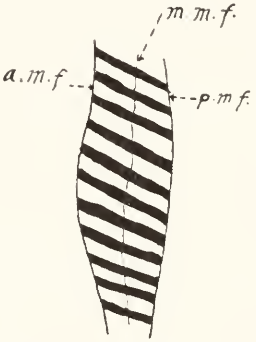


FIG. 3. Camera lucida sketch of a portion of the membranelle field at about point *m. p.* in Plate III, but taken from another specimen. *A. m. f.*, anterior membranelle fibril; *m. m. f.*, median membranelle fibril; *p. m. f.*, posterior membranelle fibril, all continued down from collar region. Silver nitrate preparation.

It is worthy of note that the apparent heavy nature of the fibrils is due to the impregnation of silver, as in material stained *intra vitam* the fibrils appear as exceedingly delicate strands.

NEUROMOTOR APPARATUS

Regarding the neuromotor apparatus, my observations tend to confirm those of Yocum except in the matter of the motorium. The fibers from the bases of all cirri except the anals were found to extend considerably farther through the cytoplasm than indicated by Yocum, but this is a matter of no special significance.

After examining literally hundreds of specimens both entire and in serial sections 2, 3, 4, 6, 8, 10, 15 and 20 microns thick, using all the techniques suggested for its demonstration, I am forced to the conclusion that there is no motorium in *Euplotes patella* as a separate and distinct body. I have never been able to see it even in specimens where the other neuromotor elements are exceptionally clear. It is quite evident in many of my preparations, both whole mount and sectioned, that the five anal cirri fibers converge, join to form one fiber and extend on into the collar without a break (Plate IV). Here the single fiber turns and runs along the anterior (outer) edge of the " collar " membranelles thence along the inner (median) edge of the " lapel " membranelles. Yocum shows the five fibers attached to the left end of the motorium (his Plate XV, Fig. 9) while the fiber to the membranelles is attached to the right end of the motorium. This makes quite a break in the continuity of the system. It is easily seen in my material that there is absolutely no jog or break in the fibers from the time they leave the cirri until they reach the membranelles. Furthermore, there is no branch line running off to any body that might be considered an attached motorium, nor is there a body which is nearby and consistently present which might be a motorium.

It is quite possible that any one of several structures in this region might be mistaken for a motorium, namely, the basal plate of one of the two or three cirri located there, groups of granules such as are found scattered through the cytoplasm, or a fold of the ectoplasm in the rather thin right peristomal phlange. Professor Yocum has kindly sent me one of his slides showing what he considers the motorium on two marked specimens. Although it is hardly just to judge from two specimens, it seems to me that the darkened area which he interprets as the motorium is a wrinkle in the ectoplasm overlying the fibers on the ventral surface. I find similar structures in my own preparations if the material is fixed on slides but not in material killed in bulk. The logical explanation of this is seen in the anatomy of the organism. In the region concerned the roof of the cytostome contains a peculiar pocket, the cytostomal recess (Plate IV). When the animal is flattened by being fixed on the slide the peristomal phlange in this region is folded by being pressed against this irregularity. Such a fold occurring regularly at this point could easily be interpreted as a motorium. Yocum's Figure 9, Plate XV, can be explained in this light if the part of the membranelle fiber before it reaches the collar be considered a continuation of the edge of the cytostomal recess. However, his Fig. 5, Plate XIV, is difficult to explain on these grounds. Such a structure might have resulted from a collection of endoplasmic granules as so frequently occurs in this species. My interpretations must be based on the fact that in my material no such body is present as that which he labels " motorium," and the membranelle fiber, instead of giving off two roots to this body, is clearly and directly continuous with the five anal cirri fibers. Serial sections stained with Mallory's triple and with the hæmatoxylins support the camera lucida drawing of the whole mount shown in Plate IV.

Rees (1930) concludes that in *Diplodinium* the object called a motorium by Sharp (1914) is only a fold of the ectoplasm, and the careful investigation that he made is quite convincing. The "motorium" of *Euplotes patella* is probably a similar structure.

Discussion

The composite picture of *Euplotes patella* shows an amazing array of fibrils which link up the organelles. This consists of a superficial network linking up bristles that protrude from the surface, and a deeper set which is associated with the motor organelles. The two are connected in the anterior region.

This picture in itself strongly suggests a sensory apparatus and a motor apparatus linked together to form an integrated coördinating system.

Klein (1926*a*) was the first to give us a detailed picture of the external fibrillar network in a ciliate. Since then he has described the "Silberliniensystem" of a number of ciliates, some of which are beautifully illustrated, some very poorly. The latter includes a distorted and almost unrecognizable *Euplotes* of undetermined species (Klein, 1928). In this paper he states that the new peristomal field (as seen in the new complex of fibers) appears before any other sign of division. Turner (1930) has shown, however, that the macronucleus is the first structure to show signs of an approaching division. In another paper Klein (1926*b*) pictures the rosettes of *E. harpa* as single granules and calls them "Basalkörner." In this paper he proposes the idea that the fibrillar network is the real coördinating system in ciliates, and in *Euplotes* it is the real neuromotor system while the system described by Yocum is contractile in function. According to him the microdissection experiments of Taylor were confused. Claiming it to be a primitive nervous system endowed with both motor and sensory functions, Klein (1929) further ascribes to the "Silberliniensystem" the power of initiating division, controlling morphogenesis and to some extent inheritance. His evidence for all this is not completely convincing.

The German workers generally consider the "classical" neuromotor apparatus as a contractile or supportive structure rather than conductile. In *E. patella* the only possible function of contractile elements would be for the operation of the cirri or membranelles, and as these motor organelles are actually groups of cilia, one would not expect them to be operated by such contractile fibers on the basis of what is known of ciliary movement. Taylor's observations show also that the anal cirri continue to function even after the fibers are cut, a fact that rather demolishes the notion that the contraction of the fibers mechanically operates the cirri. Again, the fact that no movement can be detected in the fibers when the cirri are beating is further evidence against this idea.

Without a motorium the neuromotor system of *E. patella* is still established as a definite apparatus. A coördinating *center* is a nice concept, but one which is not indispensible to a neuromotor apparatus. Taylor's results are as significant without a motorium as with, when one considers the fibers themselves as the coördinating mechanism; the continuity of the fibers from the membranelles to the anal cirri maintaining the coördination.

The function of the external fibrillar network is less well established. In *E. patella* where the cortex is so strong and unyielding it is difficult to believe that a network so delicate as that seen in living specimens could add much to the rigidity of the body. From purely morphological evidence it appears that the network is sensory in nature. It is just under the pellicle where one would expect such a system, and it connects up all the bristles which appear to be sensory elements. The joining of this system to the neuromotor fibers makes a complete conductor system—sensory and motor—that seems not only adequate to interpret the structures seen, but also to explain what is known of the reactions of the organism.

In the more primitive ciliates with cilia distributed over the body, the " silver lines " may well be both sensory and motor in function, forming a kind of primitive coördinating apparatus which controls the action of the cilia in response to stimuli received. In *Euplotes,* which is one of the most highly organized of all the Protozoa, it is not surprising to find the conductor system more or less divided up into sensory and motor departments comparable in a way to the more highly specialized members of the Metazoa.

Summary

1. The external fibrillar system or " Silberliniensystem " of *Euplotes patella* is described as a regular latticework on the dorsal surface and a more irregular network ventrally.

2. These lines are associated with rows of rosettes from which bristles protrude on both dorsal and ventral surfaces. These bristles are thought to be sensory in function and the network a sensory conductor system.

3. The neuromotor system was studied and Yocum's description supported except for the motorium. Evidence is presented which seems to indicate that there is no motorium in this species.

4. The external network is directly connected with the neuromotor system.

5. Discussion brings out the probability that the neuromotor apparatus in *E. patella* is thus augmented by a distinct but connected external network of sensory fibrils.

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SELECTIVE ACCUMULATION OF IONS IN CAVITIES INCOMPLETELY SURROUNDED BY PROTOPLASM

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Selective accumulation of ions in tissue fluids has usually been thought of as a result of more or less complete enclosure of the fluid within a continuous layer of protoplasm or plasma membrane. Accordingly, when a body fluid containing principally a few selected ions is separated from the external medium by an epithelium, the cells of this epithelium are supposed to press so closely one against the other that ions can only cross through the epithelium by traversing plasma membranes and cytoplasm. In like manner, the Casparian strips have been thought to block essentially all intercellular paths between the soil solution and the sap in the stele of the vascular plant. A study of the fluids of the marine green alga *Codium Bursa* (L.) Ag. (Order: Siphonales) shows that assumptions of this type are not necessary, since in the case of *Codium* there is selective accumulation of ions in the fluid of the central cavity, which is open to the sea water by intercellular paths.

A brief description of the plant may be given here. Its thallus is in the form of a hollow oblate spheroid a few centimeters in diameter, attached by rhizoids at the base. The walls of the spheroid are a few millimeters thick, and consist of two layers. The internal layer is made up of intertangled basal filaments running for the most part in a tangential direction. From these there arises as an external layer a dense felt of clavate radial filaments aptly referred to as " palisade cells." The walls of these cells are made up of pectins with some cellulose, and are considerably thickened at the distal ends. Thickenings on the interior of the cell walls obstruct free movement of material at various points, notably in the basal filaments close to the branching of each palisade cell. A strand of protoplasm is supposed to penetrate through these thickenings, but Arcangeli reports that pressure exerted on the filaments to one side of a thickening could not be made to force material past it. (Arcangeli, 1874.) In any event a vacuole extends continuously from near the tip of each palisade cell through at least some portion of a basal filament. Turgor of the palisade cells is marked, and although not mutually adherent, they are closely pressed together.[1] Published descriptions make no mention of canals opening to the exterior from the central cavity, although such things are said to be present in related species. To make sure about this, specimens of *Codium Bursa* were wiped dry with filter paper and then squeezed. No fluid was forced out of the plant in this way. The central cavity of *C. Bursa* has therefore no obvious direct connection with the sea water through orifices or canals.

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[1] The description is taken for the most part from F. Oltmann's *Morphologie und Biologie der Algen,* II Aufl., Jena, 1922, where further details may be found.

This makes it appear that ions can pass into the central cavity from the surrounding sea water by either of two paths, traversing, respectively, the protoplasm and the cell wall material. Whatever the path by which ions enter the central cavity, it is clear from Table I that a selective process occurs at some point, since K is present in the central cavity in higher concentration than in the surrounding sea water, while the Na concentration is lower. The halide concentration is slightly higher, presumably because SO_4, which was not separately determined, is partially excluded.[2]

TABLE I

The concentrations of K, Na, and Cl in the cell sap, and in fluid from the central cavity of *Codium Bursa* (L.) Ag., and in the surrounding sea water.

Fluid	Concentration, millimols per liter			Ratio of K to Na
	K	Na	Halide	
Cell sap	84	627	657	0.134
Central cavity	61	567	624	0.108
Central cavity	64	576	619	0.111
Sea water *	22	641	656	0.034

* The sea water was taken from the aquarium system in which the plants were immersed.

Table I also shows that sap exuded under pressure from cut edges of thallus is still more modified in the direction of selective accumulation of K than is the fluid in the central cavity. The sample of sap is presumably mixed with a good deal of sea water and other fluids, and the pure cell sap probably contains K in even higher proportion than indicated in the table.

It therefore appears probable that the selective process results during the passage of ions from the sea water into the cells, and perhaps also on passage from the cells into the fluid of the central cavity. Restricted but non-selective interchange of ions along the cell walls would account for the fact that the fluid of the central cavity is more like sea water than is the cell sap.

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[2] Halide was determined by titration with $AgNO_3$, Na + K as total sulfate, K as chlorplatinate, and Na by difference.

It must be concluded, therefore, that some accumulation of ions may occur in fluids which are only partially separated from the surrounding medium by living protoplasm. Such tissues as the endodermis of roots of vascular plants, the gill epithelia of fishes, or the epithelium of the convoluted tubules of the mammalian kidney may fail to form a continuous wall of protoplasm, and still give rise to what is known as the selective accumulation process, viz., the partial exclusion of some ions, and the movement of other ions through the tissue against their own activity gradients.

The plants for these analyses were obtained at the Stazione Zoologica at Naples, where the writer occupied the Woods Hole-Columbia University table. The analytical work was supported by a grant from the National Research Council. Grateful acknowledgment is made for these aids.

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REGENERATION IN THE CARAPACE OF DAPHNIA MAGNA

I. The Relation between the Amount of Regeneration and the Area of the Wound during Single Adult Instars

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In all animals the rates of regeneration appear to have identical characteristics. Przibram (1919) cited the experiments of eleven workers on twenty species and reported that the rates of regeneration are most rapid at the beginning of the process and decrease as regeneration continues. After a study of twelve diverse species, Zeleny (1909*a*) arrived at the conclusion that the rates of regeneration vary directly with the degree of injury up to a maximum, beyond which the rates decrease. Carrel and his colleagues (1916, etc.) found that wound closure in man follows the same general trend, in that the rate of cicatrization is proportional to the area of the wound, but diminishes less rapidly than the area.

The above-mentioned works deal with regeneration of complex structures composed of many tissues and consequently treat with a composite of the rates of individual tissues. A study of the rate in a less complex structure should lead to a better understanding of the fundamental problems involved.

The carapace of *Daphnia magna* is a comparatively simple structure. Two layers of hypodermis (Fig. 1) constitute the cellular tissue of the ventral half of the carapace posterior to the shell gland, the region which is injured by the operative procedure outlined below. Between these layers is a blood chamber and associated with them on their outer surfaces is the chitinous integument. The pillars, which probably also consist of chitin, serve as supporting structures. Figure 2 is a surface view showing the arrangement of the outer hypodermal cells. The markings of the carapace which are characteristic of this cladoceran conform closely to the cell boundaries. The pillars do not appear to be arranged in any definite order.

Sections of the regenerated portions of the carapace are identical with those of the uninjured regions. When viewed from the surface, the shape of the regenerated hypodermal cells is quite variable, in contradistinction to that of the original cells. Since the carapace remains the same in cross-section, the difference in the area of the wound from one time to another serves as a three-dimensional measure of the amount of regeneration during the interval.

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[1] The greater part of the experimental work upon which this paper is based was carried out at the Zoölogical Laboratory of the State University of Iowa.

The purpose of this paper is to present a study of the amount of regeneration in the carapace during single physiological time units, *i.e.* the instars, in relation to the size of the wound under varying conditions of age of the animal, age of the wound, and temperature. These results will be discussed in relation to those secured by others for more complex structures in other forms.

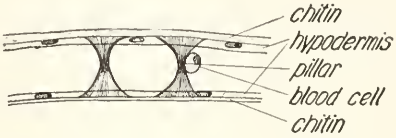

FIG. 1. Camera lucida drawing of a cross-section through the carapace.

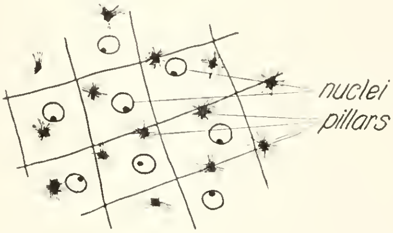

FIG. 2. Camera lucida drawing of the outer surface of the carapace showing the typical arrangement of cells. The surface markings correspond to the cell boundaries. The pillars appear in no definite order.

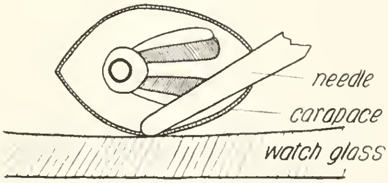

FIG. 3. Diagrammatic section through an animal showing method of operation. Injury to structures other than the carapace is avoided.

The writer wishes to acknowledge his indebtedness to Dr. L. A. Brown and Dr. J. H. Bodine—to the former for suggesting the problem, and to both for their many helpful suggestions and criticisms as the work progressed.

Materials and Methods

The material, *Daphnia magna* Straus., was secured from Banta's laboratory in 1927. Females from three clones were employed. The original clone was used in the experiments on the effect of age but died out late in 1928. A new clone, started with ephippial eggs from the former in January, 1929, was used in the experiments dealing with temperature effect. Another clone, derived from the latter, was used in the experiments concerned with age of the wound.

Individuals were isolated within twenty-four hours after their release from the brood chamber of the mother and placed in vials containing fresh manure-soil medium (Banta, 1921). The vials used in the experiments on the effect of age and of temperature contained thirty to thirty-five cc. of medium, while those used in the experiments on the effect of the age of the wound contained fifty to sixty cc. of medium. Semi-weekly throughout the experiments one-third of the fluid in each vial was removed and replaced by fresh culture medium.

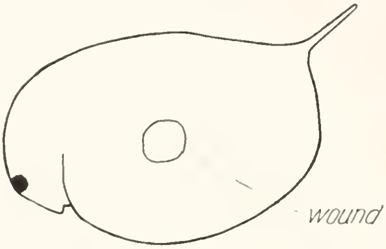

Fig. 4. Outline drawing showing position of wound in carapace.

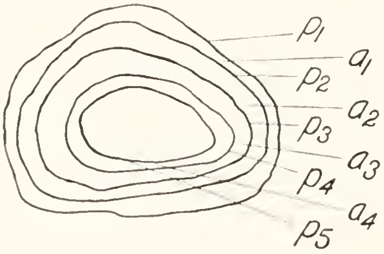

Fig. 5. Camera lucida drawings of a wound during five successive instars. p_1 represents the edge of the wound during the first post-operative instar; p_2, the second; etc. The area a_1 represents the amount of regeneration during the first post-operative instar; a_2, the second, etc. In the graphs which follow, the amount of regeneration during each instar is plotted against the area of the wound during that instar, *i.e.*, a_1 against total area enclosed by p_1; etc.

In the experiments to determine the effect of the temperatures 15° C. and 25° C. the animals were placed, immediately after isolation from the mother, in an electrical refrigerator fitted with a mercury thermoregulator, a heating element, and an electric fan (air temperature ± 0.5° C.). All other experiments were carried on at room temperature (18°–25° C.).

In the operative procedure the animals were placed in watch-glasses and immobilized with a chloretone solution of a concentration just sufficient to bring about cessation of movement. The chloretone did not appear to have any detrimental effects. Part of the area of the carapace in contact with the watch-glass was crushed by applying a steel needle to its inner surface (Fig. 3). Figure 4 shows the position of the wound. The proportions of the wounds were varied by using needle points of different sizes and shapes. Approximately 4000 individuals were operated upon in these experiments.

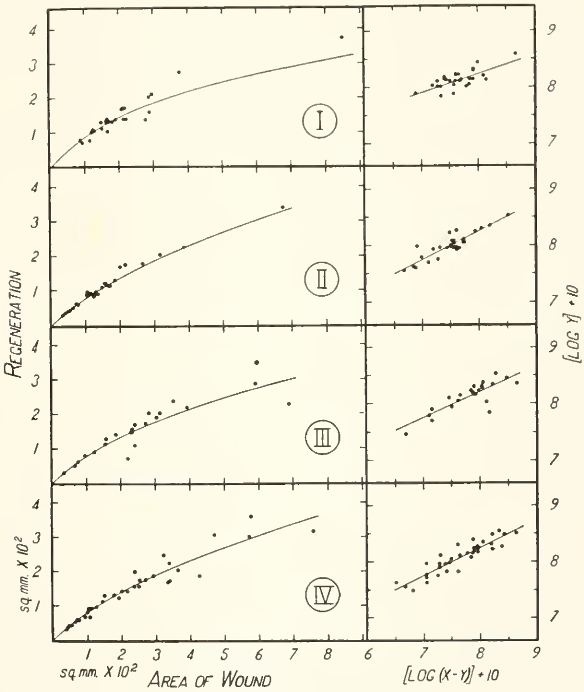

FIG. 6. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instars I–IV. Each animal was operated early in the instar before the one in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

For comparison with later adult instars see Fig. 8.

During the early part of each instar following operation the animals were again immobilized with chloretone and camera lucida drawings (175 ×) made of the wounds. From these drawings the areas were determined by the use of compensating polar planimeters (Keuffel & Esser Co., No. 4242 and No. 4240) and the perimeters by a measuring wheel (Keuffel & Esser Co., No. 1694A) graduated to 1/32 inch.

EXPERIMENTAL

A few hours after an injury is inflicted on the carapace of *Daphnia magna* a brown material forms at the edge of the damaged area. In all probability this brown material is clotted blood in which the tyrosine has been oxidized (Pinhey, 1930; Anderson, 1932*b*). The brown ma-

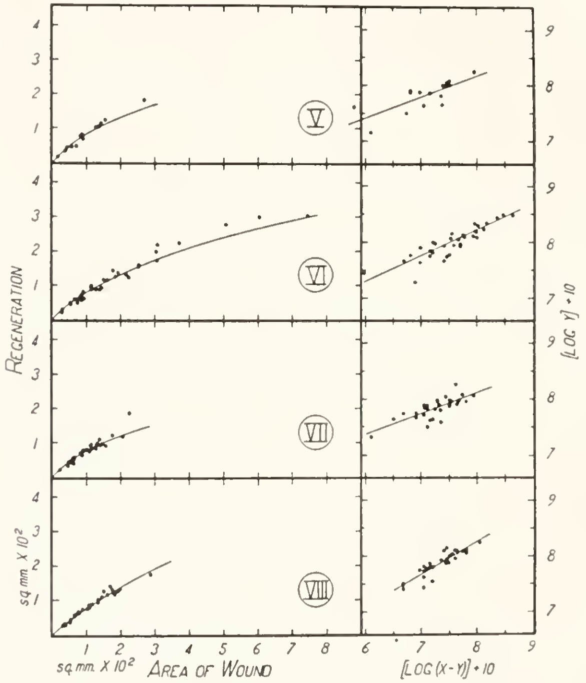

FIG. 7. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instars V–VIII. Each animal was operated early in the instar before the one in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

For comparison with other adult instars see Fig. 8.

terial is shed with the chitin at the next ecdysis when the wound is made before three-fourths of the instar has passed. However, if the injury is made after three-fourths of the instar has passed, the brown material is retained until the second molt following the injury. If an injury is inflicted early in the instar the edge of the wound during the next instar is smooth and clear. If the injury occurs after half but before three-fourths of the instar has passed, the edge of the wound is rough during the following instar. These phenomena appear to be directly related to the presence or absence of a second layer of chitin at the time of injury (Anderson and Brown, 1930). In all cases, no matter what be the nature of the wound during the first post-operative instar,

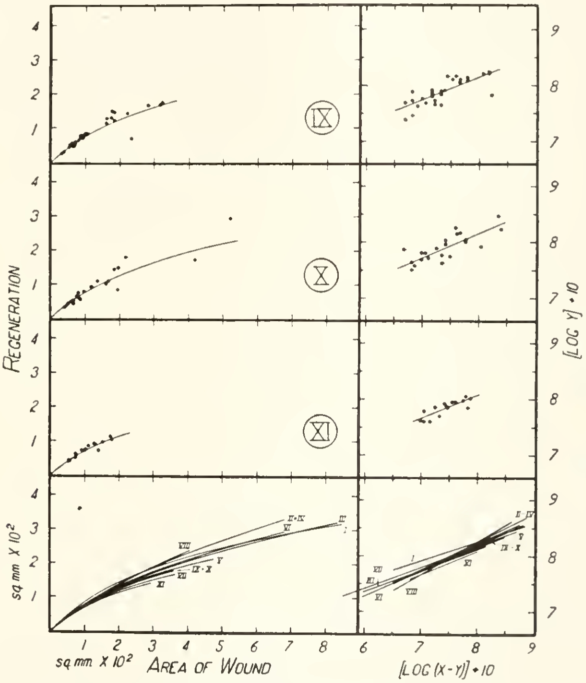

FIG. 8. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instars IX–XI. Each animal was operated early in the instar before the instar in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

The bottom graphs give the curves for each of the adult instars I–XI for purposes of comparison.

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the edge of the wound is smooth and clear throughout the remaining instars, *i.e.* until complete closure results.

Further, when an operation is performed within the first half of the instar some regeneration takes place before the animal molts. The area of the wound during the following instar is less than that included within the brown ring during the instar of operation. If an operation is performed within the period after half the instar has passed and before three-fourths is over, the wound during the next instar is approximately the same size as the area enclosed by the brown material during the operative instar. In case the injury is produced after three-fourths of the instar has passed, the brown material occupies the same area during the next instar as it does during the instar of injury. Normally wounds diminish noticeably in size by the second post-operative instar irrespective of the time within the instar of operation that the injury is inflicted.

TABLE I

Values of the constants in the equation $y = a(x - y)^k$ expressing the relation between the amount of regeneration (y) and the area of the wound (x) for first post-operative instars with reference to the age of the animals. The data on which these constants are based are shown in Figs. 6, 7, and 8.

Adult instar	Constants		Number of cases
	a	*k*	
I	.09	.35	29
II	.18	.50	29
III	.13	.46	23
IV	.18	.50	35
V	.09	.38	16
VI	.12	.45	34
VII	.07	.37	31
VIII	.27	.59	30
IX	.09	.40	30
X	.10	.43	23
XI	.08	.42	15

The wound remains the same in size and shape throughout any one instar. Change takes place only at ecdysis and shortly thereafter. This is also true for the body length and for the antennal segments of the animal (Anderson, 1930, 1932*a*, *b*) and for the antennæ of *Simocephalus gibbosus* and *Daphnia carinata* (Agar, 1930).

Preliminary experiments showed the impracticability of securing wounds of an exactly identical size and shape. The area injured was always greater than the immediate area crushed. This extended area of injury varied considerably for each case. These experiments also showed that the amount of regeneration during any instar varied with the size and shape of the wound. The shapes may be classified according to the ratios of the perimeter to the square root of the area. The data included in this paper are for wounds where the ratio of the perimeter to the square root of the area does not exceed four. The wounds coming under this classification range from approximate circles to elongated ovals whose lengths rarely exceed twice their widths.

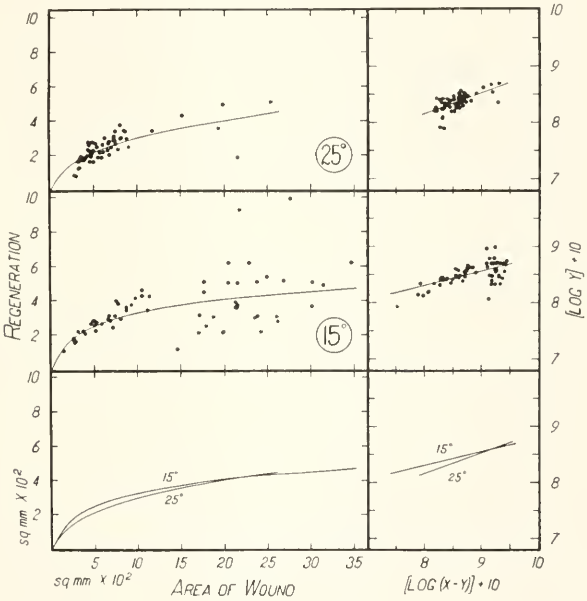

Fig. 9. Graphs showing the relation of the amount of regeneration to the area of the wound during adult instar II at the temperatures 15° and 25° C. Each animal was operated early in the instar before the one in question. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

With the foregoing facts in mind, a series of experiments was designed to determine the amount of regeneration for wounds of different sizes but of the same approximate shape, controlled by the means outlined above, during each of the first eleven adult instars. In each case the animals were operated early in the instar previous to the one for which records were taken. The results are shown in Figs. 6, 7, and 8. The area of the wound represents size during the instar in

TABLE II

Values of the constants in the equation $y = a(x - y)^k$ expressing the relation between the amount of regeneration (y) and the area of the wound (x) for first post-operative instars with reference to temperature, the age of the animals being the same. The data on which these constants are based are shown in Fig. 9.

Temperature	Constants		Number of cases
	a	k	
° C.			
15	.06	.25	62
25	.08	.36	56

question. The amount of regeneration is the difference between the area of the wound during the instar in question and the area during the following instar (Fig. 5). The results indicate that the amount of regeneration for wounds of identical sizes is approximately the same during the adult instars studied.

Mere graphic presentation of the data yields only a rough means of comparing the results. It was therefore deemed advisable to determine the nature of the relation between the amount of regeneration and the area of the wound. On first glance the relation seems parabolic. The simplest expression for a parabolic curve would be

$$x = ay^k. \tag{1}$$

But the amount of regeneration can never exceed the area of the wound. By adding y to the second member of equation (1) we have

$$x = ay^k + y. \tag{2}$$

By letting y be the amount of regeneration and x be the area, the amount of regeneration can never exceed the area of the wound.

For ease of manipulation, since both x and y are known, equation (2) was converted to

$$y = a(x - y)^k. \tag{3}$$

If this equation is suitable, double logarithmic plots of the amount of regeneration (y) against the area minus the amount of regeneration ($x - y$) should yield a straight line.[2] Such is practically the case as is shown in Figs. 6–11.

At present this equation may be considered only descriptive. No biological significance has been assigned to the constants.

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[2] It may be noted that $(x - y)$ is the area of the wound after y has been regenerated.

The constants for each experiment were determined by the method of least squares. The curves in the figures were fitted by means of the constants so determined.

A study of the composite graphs in Fig. 8 and the constants in Table I shows that in general for wounds of the same size the amount of regeneration decreases as the animals grow older.

The amount of regeneration for wounds of identical sizes and shapes is less during the pre-adult instars than during adult instars. The amount of regeneration increases with each instar until the adult condition is reached. The first adult instar is the one during which the first clutch of eggs appears in the brood chamber. Considerable irregularity has been found in the number of pre-adult instars for this

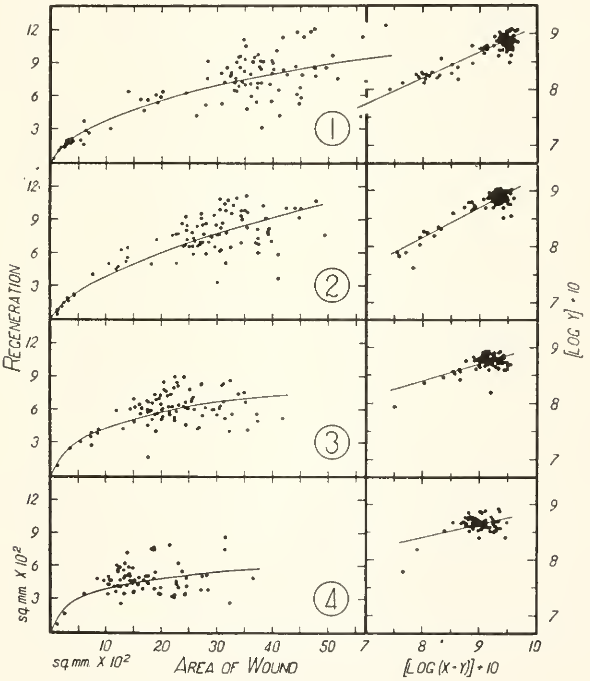

FIG. 10. Graphs showing the relation of the amount of regeneration to the area of the wound for each of the first four instars after operation. Each animal was operated early in the first adult instar. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

For comparison with later instars see Fig. 11.

species (Anderson, 1930, 1932*a*). Consequently comparisons of the amounts of regeneration for any given pre-adult instar are hardly justifiable at present.

Another series of experiments was carried out to test the effect of temperature on the amount of regeneration during a single instar. Animals which had been kept at specified temperatures (15° and 25° C.) from the time of their isolation were operated during the early part of the first adult instar. Records were taken for the second adult instar. The results are shown in Fig. 9. The constants are given in Table II. From the figure it may be seen that the difference in temperature within the limits studied has relatively no effect on the amount of regeneration.

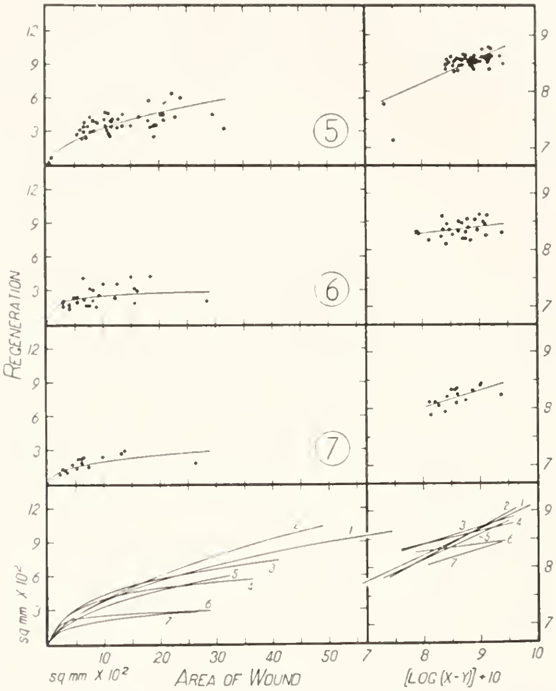

FIG. 11. Graphs showing the relation of the amount of regeneration to the area of the wound for the fifth to the seventh instars after operation. Each animal was operated early in the first adult instar. The curves were fitted by means of the equation:

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants. The logarithmic plots show the relation between y and $(x - y)$. Each point represents an individual case.

The bottom graphs give the curves for each of the first seven instars following operation.

The preceding experiments were concerned with relatively small wounds and with the first post-operative instar. Another series of experiments was designed to determine the amount of regeneration for the largest possible wounds and further to determine the effect of the age of the wound on the amount of regeneration. Animals were operated early in the first adult instar and records taken during each successive instar until death ensued. The results are shown in Figs. 10 and 11. The size of the wound is always that during the instar in question as shown in Fig. 5. The results for the eighth and ninth post-operative instars are not shown, since the number of cases was considered too small to be of significance. The values of the constants for each post-operative instar are given in Table III.

TABLE III

Values of the constants in the equation $y = a(x - y)^k$ expressing the relation between the amount of regeneration (y) and the area of the wound (x) for successive instars following operation. The data on which these constants are based are shown in Figs. 10 and 11.

Post-operative instar	Adult instar	Constants		Number of cases
		a	k	
1	II	.14	.45	105
2	III	.17	.54	102
3	IV	.10	.30	94
4	V	.08	.23	79
5	VI	.11	.45	51
6	VII	.03	.12	28
7	VIII	.04	.30	15
8	IX	.03	.25	10
9	X	.06	.47	5

The results show that the relation tends to be the same for large and for small wounds, during the first post-operative instar. The values of the constants for the first few post-operative instars are almost the same as those where the age of the animal alone was considered. A study of the composite graph in Fig. 11 shows that the amount of regeneration decreases with the increase in the number of instars between the time of operation and the instar during which records were taken and to a greater extent than that where age of the animal alone is considered.

DISCUSSION

Zeleny (1909*a*) pointed out the principal sources of error that arise in quantitative work on regeneration. As he listed them they are: (1)

age, (2) periodic physiological changes, (3) character of the laboratory histories, (4) changes in the rate during the regeneration period, (5) successive regenerations, (6) level of the cut, (7) temperature, (8) food, (9) differences in manipulation, (10) departure of living conditions from the optimum, (11) the relation of the degree of injury to the optimum degree, (12) individual variation. Five of these factors have been made subjects of the present study, namely: age, periodic physiological changes—the instar, changes in rate during the regeneration period, temperature, and relation of the degree of injury to the optimum degree. The other factors have been eliminated in the following manner:

The Character of the Laboratory History.—The animals are well adapted to laboratory conditions since they have been reared in laboratories for well over a decade.

The Level of the Cut.—Zeleny had reference to the amount removed. This factor may be thought of in a somewhat different sense in the present case, for the various regions of the carapace may have different rates of regeneration. The positions of the wounds were as nearly identical as possible in all instances.

Successive Regeneration.—The problem at hand is not concerned with this factor.

Food.—The food used was as uniform as possible. Banta's manure-soil medium was employed in all experiments.

Differences in Manipulation.—As far as possible all individuals in any one series of experiments were subjected to the same manipulations. (See *materials and methods.*)

Departure of Living Conditions from the Optimum.—All animals in any one series of experiments were subjected to the same living conditions. The animals retained their vigor as was evidenced by the continued production of young throughout the experiments.

Individual Variation.—This factor was reduced to a minimum through the use of animals from a single clone in each series of experiments, thus insuring individuals genotypically alike. Large numbers of animals were employed as may be seen from the tables.

Zeleny in his treatment of the above factors suggests that the molting period be used as the center of observations for Crustacea. Indeed, such is the only possible way to handle the above problem. The size and shape of the animals remain constant throughout any one instar. Only at ecdysis and within the period immediately following, during which the chitin hardens, does any normal change take place. The wound remains the same in size as does the carapace of the animal.

Whether or not adult instars are quantitatively equivalent units is a question which remains unsolved. Under optimum conditions females bear a brood of young during each adult instar. At the beginning of each adult instar a number of eggs are passed into the brood chamber. These develop into free-swimming young which are released a few hours before the end of the instar. Usually the time between the release of the young and the end of the instar increases with the age of the animal. The duration of an instar also probably increases with age.

MacArthur and Baillie (1929) and Obreshkove (1930), working with *Daphnia magna* and *Simocephalus expinosus* respectively, found that the metabolic rate decreases with age. If adult instars are quantitatively equivalent units one would expect that the instars would be proportionately longer as the metabolic rate decreases. However, since the amount of regeneration is somewhat less for wounds of the same size during later adult instars than for earlier, one might come to the conclusion that instars are not necessarily equal. Further work on instar length in relation to metabolic rate is necessary to determine the status of the adult instars as quantitatively equivalent units.

Zeleny (1909*a*) found that the rate of regeneration of an organ increases with the degree of injury up to an optimum beyond which the rate decreases. In his experiments several organs were removed and the rate of regeneration for one was compared to the rate when that one only was removed. One must note in the present results that the amount of regeneration for any instar increases with the size of the wound, but the ratio of the amount of regeneration to the area of the wound decreases as the area is increased.

Zeleny (1909*b*) also noted the rate of regeneration for younger animals to be less than for older, but the actual time necessary to replace an organ to be more for older than for younger animals. Du Noüy (1916) showed that the rate of cicatrization in man decreased with the age of the individual. In the case at hand the amount of regeneration during the first post-operative instar for wounds of equal size in adult animals is more in younger than in older animals.

Przibram (1926) has regarded regeneration as an acceleration of normal growth processes. This viewpoint is tenable if the rate of regeneration need not be dependent on the rate of normal growth. Increase in size of *Daphnia magna* females is greatest during the first two adult instars and becomes less with each adult instar up to the tenth, after which time the size of the animals remains relatively constant (L. A. Brown, unpublished data). It may be observed in Fig. 8 that the amount of regeneration decreases with age. The decrease in the amount of regeneration is not equivalent to the decrease in the amount of growth. Godlewski and Latinik (1930) reported that in the tail of the axolotl growth and regeneration are largely independent of each other.

Durbin (1909) found that the rate of regeneration in tadpole tails was quite slow at first but increased to a maximum and decreased to zero after a time. Przibram (1919) concluded from experiments on twenty species from five different phyla that the rates of regeneration are most rapid at the beginning of the process and decrease as regeneration continues. A study of Figs. 10 and 11 shows that the amount of regeneration is greatest at first and decreases as the process continues.

The results here presented agree in general with the findings of Carrel and Hartmann (1916) for the cicatrization of wounds in man, in that the rate is proportional to the area of the wound.

Ebeling (1922) reported that the value of the Q_{10} was 2 for the rate of cicatrization of wounds in alligators. The value of the Q_{10} for the instar length of young adult female *Daphnia magna* is approximately 2.3 between the temperatures 15° and 25° C. (Anderson and Brown, 1930). While instar length varies directly with the temperature, the amount of regeneration is relatively unaffected. The rate of regeneration taken on the basis of absolute time units would therefore be affected by temperature to the same degree as is instar length.

One may conclude that the amount of regeneration during any single adult instar is a parabolic function of the area of the wound during that instar, and is independent of the temperature, but decreases with the age of the animal and to an even greater extent with the age of the wound.

SUMMARY

1. This report deals with a study of the amount of regeneration in the carapace of *Daphnia magna*.

2. The amount of regeneration during any adult instar is a parabolic function of the area of the wound during that instar and may be expressed by the equation

$$y = a(x - y)^k,$$

where y is the amount of regeneration; x, the area of the wound; and a and k constants.

3. The amount of regeneration during any adult instar decreases with the age of the animal.

4. The amount of regeneration during adult instars is independent of the temperature.

5. The amount of regeneration during any adult instar decreases with the age of the wound but to a greater extent than when the age of the animal alone is considered.

6. These results have been discussed with reference to those secured by others for relatively complex structures.

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MITOSIS IN HYDRA

Mitosis in the Ectodermal Epithelio-Muscular Cells of Hydra

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Introduction and Historical

Various workers with *Hydra* have come to the conclusion that the ecto-epithelio-muscular cells of *Hydra* do not divide, and that if mitotic figures are found in them it is the exception rather than the rule. Downing (1905) says, "In all the sections studied mitosis has been the universal mode of division in the interstitial cells, the exceptional mode in the ectoderm cells and amitosis the constant rule in the endoderm cells. Fig. 5 shows an ectoderm cell in process of amitotic division. At first it was thought this was the only mode of division of the ectoderm cells. But a few cases have been observed, only two or three in hundreds of sections, however, where mitosis occurred in an undoubted ectoderm cell. Schneider had observed and figured mitosis in an ectoderm cell. But cell multiplication, except of the interstitials seems to be almost invariably amitotic." (Pages 411–412.)

Strelin (1928) says, "K. C. SCHNEIDER (1890) findet in seiner eingehenden zytologischen Untersuchung gar keine Übergangszellenformen zwischen den I. Z. einerseits und den Epithelmuskelzellen andererseits; deshalb ist er geneigt zu denken, das die Neubildung der Epithelmuskelzellen auf Kosten der Teilung der differenzierten Elemente stattfindet. Als Bestätigung führt SCHNEIDER die Teilungsbilder der Epithelmuskelzellen an.

"Vielen Autoren ist es aber gar nicht gelungen, in den spezialisierten Zellen der Hydra Mitosen zu beobachten; ich konnte ebenfalls in diesen Zellen kein einziges Mal Mitosen beobachten, obgleich ich zu diesem Zwecke eine grosse Zahl von Schnitten durchgesehen habe. SCHNEIDER (1890). STECHE (1911), GELEI (1924), MATTES (1925), KANAJEIO (1926) u.a. haben in den zpezialisierten Zellen Mitosen angetroffen, sie halten aber dieselben für eine seltene Erscheinung. Es ist daher kaum möglich, die Neubildung der Epithelzellen ausschliesslich durch die Vermehrung derselben zu erklären." (Page 274.)

We see then that Strelin maintains that the indifferent cells are transformed into ecto-epithelio-muscular cells in mitosis. He further states, "Die Frage darüber, auf welche Weise aus einer ganzen Gruppe kleiner junger Epithelzellen nur mehrere Elemente entstehen, ist für mich nicht ganz klar, um so mehr, als die Zahl der Zellen sich stets durch Vermehrung vergrössern muss. Es ist möglich, dass im gegebenen Falle die Rückentwicklung der jungen Epithelzellen in Interstitiellzellen stattfindet; es ist aber viel wahrscheinlicher, dass einige junge Epithelzellen auf den Anfangsstadien ihrer Entwicklung aufgehalten werden. In den grösseren von den jungen Epithelzellen kommen ebenfalls, wie oben vermerkt wurde, Mitosen vor; man muss aber bemerken, dass je mehr die Zellen differenziert sind, desto seltener in ihnen Mitosen vorkommen; in den erwachsenen Epithelzellen ist es mir niemals gelungen, wie ich bereits erwähnt habe, Mitosen zu begegnen." (Pages 278–279.) We see further, that Strelin noticed the difference in the size of the indifferent cells, but he calls the larger of these, which he found in mitosis, "jungen Epithelzellen" and thinks that when these "jungen Epithelzellen" are further transformed into ecto-epithelio-muscular cells they never divide.

McConnell (1930 and 1932) has demonstrated the mitosis of the endo-epithelio-muscular cells and made reference to the mitosis of the ecto-epithelio-muscular cells. Owing to the fact that so many mitotic figures have been found (and easily found) in the ecto-epithelio-muscular cells, it was decided to make a study of this process to clarify the question of mitosis in these cells.

MATERIALS AND METHODS

Pelmatohydra oligactis was used in this study. The material was collected in the vicinity of the University of Virginia, Charlottesville, Virginia, U. S. A.; the University of Ljubljana, Ljubljana, Jugoslavia; the Kaiser Wilhelm-Institute for Biology, Berlin-Dahlem, Germany. Preparations studied were as follows: without buds 105, with buds 75, with testes 15, with ovaries and testes 7, with ovaries 3. These *Hydra* were killed and fixed in the following solutions: in Bouin's, in Fleming's, in Carnoy's acetic alcohol, and in Zenker's. Sections were cut at five and seven microns. Staining was done on the slide with iron-hematoxylin and acid fuchsin, with Delafield's hematoxylin, and Bordeaux red; methyl violet and water-soluble eosin were also used in some instances. The preparations were mounted in Damar and Canada Balsam. They were studied with a compound binocular microscope equipped with 430 × dry and 97 × oil immersion objectives; 5 ×, 10 ×, and 15 × paired oculars were used. Microphotographs were made at magnifications of 430 ×, 860 ×, and 970 ×, during which processes an Orsam point-light was used.

PROPHASE

During the prophase the nucleus enlarges and moves slightly toward a central distal position; it takes nuclear stains much more intensely than do the normal resting nuclei. (See Fig. 3, Plate I.) The number of nucleoli varies from one to four, and they remain evident up until the beaded spireme is formed. The entire cell rounds up and the muscular processes are withdrawn from the mesoglea. This rounding-up process gives a characteristic swollen appearance to the area where the mitosis is taking place. The beaded spireme is very active in the nuclear area and may be seen, in maceration preparations, to be very actively whirling about. There are twelve chromosomes of V-shape.

METAPHASE

The chromosomes line up on the equatorial plate with the tips of the V's pointed to the center. The asters and centrioles are quite prominent. The chromosomes start dividing at the tip of the V, and continue dividing longitudinally. As the open arms of the daughter chromosomes recede, they remain connected by spindle fibers.

ANAPHASE

As the daughter chromosomes approach the division centers the tips of the V's fuse slightly, with the ends of the daughter chromosomes protruding in various directions. As they move towards the pole the centriole becomes more and more indistinct and gradually disappears.

PLATE I

1. Resting nucleus with four nucleoli. Bouin's; iron-hematoxylin. 776 ×.

2. Early prophase to the left, and daughter cell to the right. Bouin's; iron-hematoxylin, and acid fuchsin. 776 ×.

3. Lower left, normal resting nucleus; upper right, later stage of prophase. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.

4. Prophase just prior to the disappearance of nuclear membrane. Bouin's; iron-hematoxylin. 776 ×.

5. Same as No. 4. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.

6. The metaphase chromosomes. Bouin's; iron-hematoxylin. 776 ×.

7 and 8. Two metaphase stages. Notice the slight bulge which makes this stage very noticeable. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.

9. Metaphase from the base of tentacle. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.

10. Metaphase. Fleming's; iron-hematoxylin and acid fuchsin. 776 ×.

11. Metaphase. Carnoy's acetic alcohol; iron-hematoxylin and acid fuchsin. 776 ×.

12. Metaphase perpendicular to mesoglea. Zenker's; iron-hematoxylin and acid fuchsin. 776 ×.

Plate I

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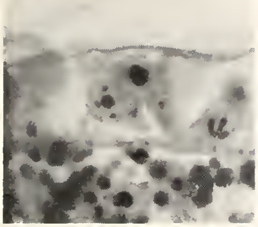

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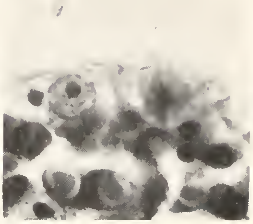

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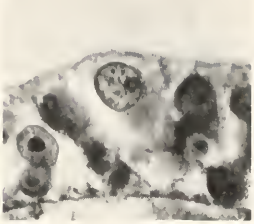

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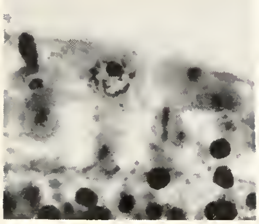

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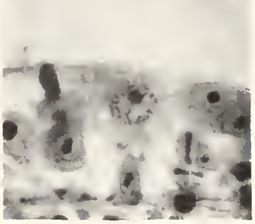

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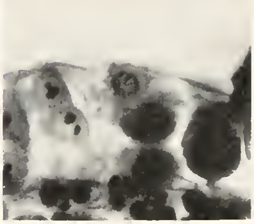

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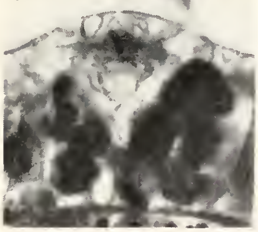

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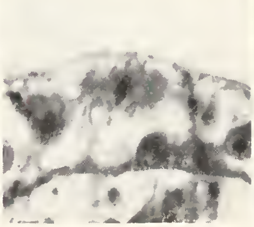

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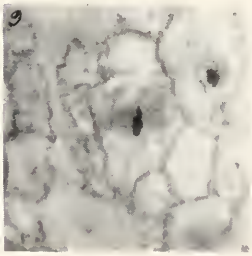

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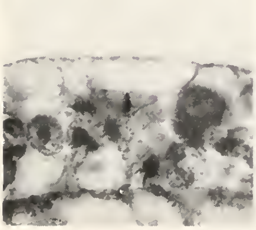

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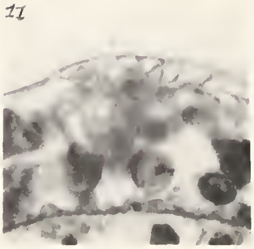

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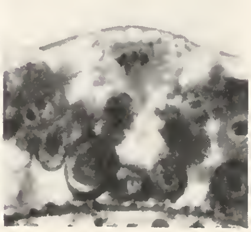

PLATE II

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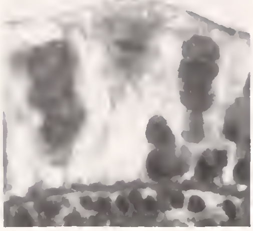

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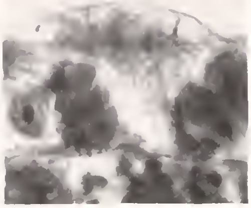

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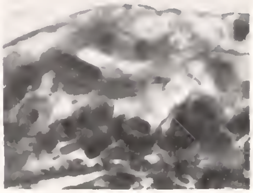

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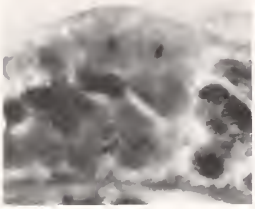

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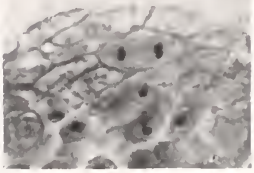

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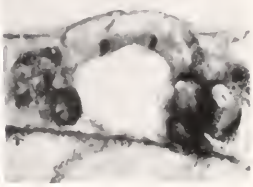

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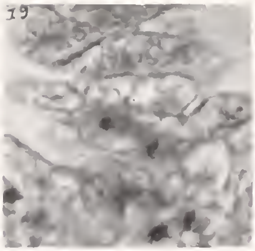

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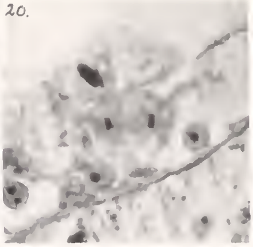

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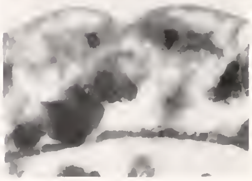

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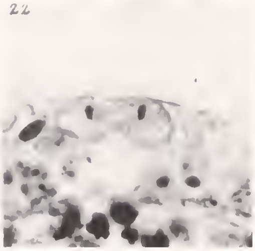

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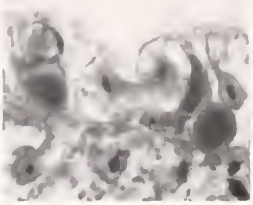

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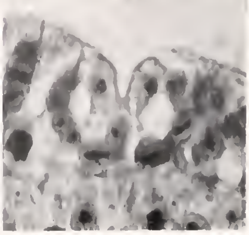

TELOPHASE

Upon reaching the area where daughter nuclei will be formed the chromosomes expand very quickly and reconstruct nuclei. The cleavage furrow is most noticeable in mitotic figures in areas where the cells are not closely crowded together or when the animal has been killed and fixed in an expanded condition. After the daughter cells are separated and the nuclei are in the process of being reconstructed, that part of the cell which lies nearest the mesoglea begins to push its way down between the surrounding cells toward the mesoglea; and, when this is reached, forms muscular processes.

TABLE I

	No. of preparations	Prophase		Metaphase		Anaphase		Telophase	
		early	late	early	late	early	late	early	late
With ovaries and testes	7	12	13	11	10	2	4	9	6
With ovaries	3	4	9	17	12	6	2	14	27
With testes	15	42	17	31	19	4	9	20	14
With buds	75	92	27	46	24	4	17	29	34
Without buds	105	101	47	30	21	7	27	49	10
Total	205	251	113	135	86	23	59	121	91

OCCURRENCE OF MITOSIS

As stated above, two hundred and five preparations were studied; Table I, showing the different stages, would seem to indicate that mitosis is the rule rather than the exception. Thus we find a total of 879 mitotic figures in 205 preparations.

PLATE II

13. Metaphase perpendicular to mesoglea. (Compare with No. 7, Plate I.) Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
14. Anaphase. Fleming's; Delafield's hematoxylin. 776 ×.
15. Anaphase. Carnoy's acetic alcohol; iron-hematoxylin and acid fuchsin. 776 ×.
16. Anaphase. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
17. Anaphase. Fleming's; iron-hematoxylin. 688 ×.
18. Anaphase. Note large vacuole between achromatic figure and mesoglea. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
19. Anaphase from base of tentacle. Bouin's; Delafield's hematoxylin. 776 ×.
20. Anaphase. Bouin's; iron-hematoxylin. 688 ×.
21. Telophase. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.
22. Late anaphase. Fleming's; iron-hematoxylin. 688 ×.
23. Telophase. Zenker's; Delafield's hematoxylin. 776 ×.
24. Telophase from tip of growing bud. Fleming's; iron-hematoxylin and acid fuchsin. 776 ×.

These mitotic figures are found in all parts of the *Hydra's* body except in the epithelio-muscular cells of the tentacles. The number of mitotic figures varies greatly as to the region of the body; the majority are found in all cases in the upper two-thirds of the body; however, many may be found in the so-called "stalk-region" and on the peristome. These dividing cells may also be found in the basal disc, but this appears to be rather rare.

In the majority of cases, the achromatic figure during the metaphase is parallel to the mesoglea; however, in some exceptional cases, one finds the metaphase spindle arranged perpendicularly to the mesoglea, in which case one of the daughter cells is buried under the surrounding epithelial cells; the fate of these buried cells has, as yet, not been determined. (Fig. 12, Plate I, Fig. 13, Plate II, and Fig. 36, Plate III.)

DISCUSSION

Kanajew (1930), who worked quite extensively on the problem of regeneration in *Hydra*, and who reviewed all of the literature on regeneration, says: "Mitosen sind nur in den I-Zellen häufig. In differenzierten Ekto- und Endodermzellen habe nur seltene Falle der Zellvermehrung bemerkt." Since Kanajew was working on regenerating *Hydra* and not *Hydra* in their normal condition, it is quite natural that he should not find many cases of the mitosis of the ecto- and endo-epithelio-muscular cells. The indifferent interstitial cells, as he noted, are found in mitosis in *Hydra* under all conditions of experimentation.

Threlkeld and Hall (1928), in their experiments upon *Hydra* under determined ion concentration, starved *Hydra* over long periods of time.

PLATE III

25. Telophase. Zenker's; iron-hematoxylin. 776 ×.

26. Telophase. Carnoy's acetic alcohol; Delafield's hematoxylin. 776 ×.

27. Telophase from tip of bud. Note remains of spindle fibers. Bouin's; iron-hematoxylin. 688 ×.

28. Telophase. Note remains of spindle fibers. Bouin's; Delafield's hematoxylin. 688 ×.

29. Late telophase, from peristomal region. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.

30, 31, and 32. Stages at which the daughter cells begin growing down toward the mesoglea, where muscular processes will be formed. Bouin's; Delafield's hematoxylin and Bordeaux red. 776 ×.

33. The two daughter cells resulting from the division of one of the mucus-secreting cells of the basal disc. Bouin's; iron-hematoxylin and acid fuchsin. 776 ×.

34. A cross-section through the lower third of a *Hydra*'s body, showing a telophase at *A*, and a metaphase at *B*. Bouin's; iron-hematoxylin and acid fuchsin. 344 ×.

35. The result of division of an epithelio-muscular cell when the metaphase was not parallel to the mesoglea. Bouin's; iron-hematoxylin. 344 ×.

36. Same as No. 35. The buried cell, *B*, has arisen from the cell *A*. 776 ×.

Plate III

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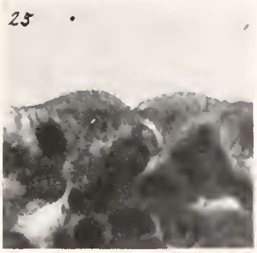

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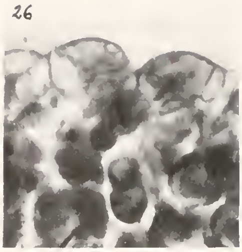

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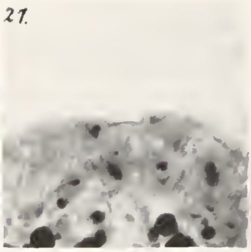

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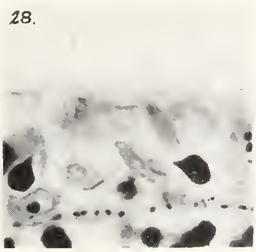

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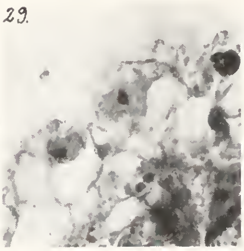

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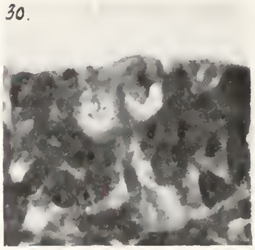

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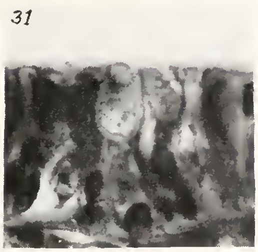

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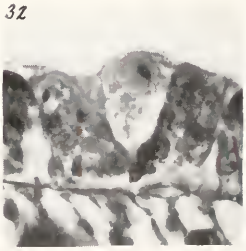

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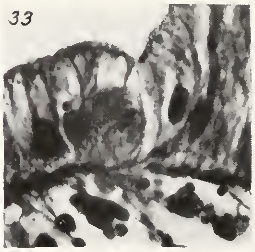

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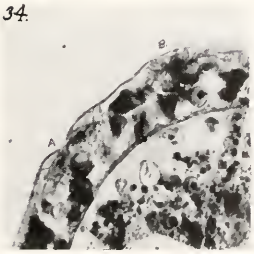

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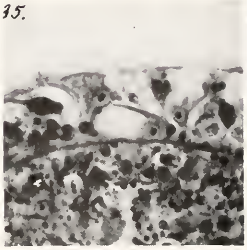

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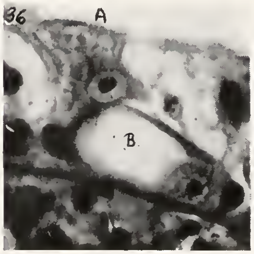

A close study of one of these preparations (*Hydra* starved 19 days), loaned to me by one of the authors, revealed the following figures given in Table II.

Table II

	Prophase	Metaphase	Anaphase	Telophase
Ecto-indifferent	5	8	3	1
Secretory cells	1	1	0	0
Endo-epithelio-muscular	0	0	0	0
Endo-indifferent	0	0	0	0
Ecto-epithelio-muscular	2	1	0	2

We see from Table II then that the division of the cells of *Hydra* may take place under the most adverse circumstances, namely, reduction and resorption, and starvation.

It appears from all the material studied that mitosis is a normal process in the ecto- and endo-epithelio-muscular cells of *Hydra.* It must be admitted that occasionally one finds nuclei which might give the appearance of having arisen by amitosis; but these cases are so rare and so difficult to account for, that they can best be omitted in the present discussion. It may be noted in passing however, that these, which appear to be amitotic divisions, sometimes occur side by side with cells which are going through the process of mitotic division.

If we assumed that the epithelio-muscular cells did not divide but were eventually replaced by cells which were elaborated from the indifferent cells, we should constantly find the indifferent cells of the ectoderm in the rather constant process of enlarging to almost four times their normal size; developing a muscular process with a myoneme; and developing large vacuoles, all of which are characteristic of the ecto-epithelio-muscular cells. If we assumed that the endo-epithelio-muscular cells did not divide we should find the various stages of the endo-interstitial cells in which they are developing muscular processes, food vacuoles, and flagella; but this is not true of any of the material studied so far, for at no time were there found any indifferent cells which might even be mistaken or described as assuming any of the foregoing characteristics of the endo- or ecto-epithelio-muscular cells. It seems then that these endo- and ecto-epithelio-muscular cells are self-propagating by the process of mitosis and that the endo- and ecto-indifferent cells are concerned not with replacing them, but with other processes, namely, with the formation of sperm, eggs, nematocysts, and nerve net cells of the ectoderm; and the formation of nerve cells, secretory cells of the general endoderm, and peristomal gland cells of the endo-peristomal region.

SUMMARY

1. The ecto-epithelio-muscular cells of *Hydra* divide by mitosis.

2. A centriole and asters are present. There are 12 V-shaped chromosomes.

3. These mitotic divisions take place in *Hydra* under all conditions.

4. There is no evidence that the indifferent interstitial cells of the ectoderm or endoderm are elaborated into the large epithelio-muscular cells.

ACKNOWLEDGMENTS

I wish to thank Dr. Jovan Hadži of the Zoological Institute, University of Ljubljana, Ljubljana, Jugoslavia; Dr. Max Hartmann, of the Kaiser Wilhelm-Institute, Berlin-Dahlem, Germany; Dr. Fauré-Fremiet of the Collège de France, Paris, France, for their aid and kind criticism while preparing this paper as a National Research Fellow, under their direction.

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MITOSIS IN HYDRA

Mitosis of the Secretory Cells of the Endoderm of Hydra

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Introduction

Schneider (1890) described the secretory cells of the endoderm of *Hydra* as follows: "Die Drusenzelien haben 2 oder 3 Geisseln, sind kurz oval und selten am basalen Ende mit einer Verlängerung versehen. Sie enthalten im Innern ein glänzendes Sekret, das in rundlichen Ballen das Protoplasma erfüllt. Der Kern liegt am basalen Ende. An der Mundscheibe haben sie rundliche Gestalt und zeigen das Protoplasma reducirt auf ein dünnmaschiges Netz. An den Tentakeln fehlen sie. Sie bilden sich aus dem subepithelialen Gewebe." (Page 363.)

Burch (1928), who studied the endodermal flagella, says: "The secreting cells of the general endoderm are club shaped with the smaller end directed toward the mesoglea. The distal end bears one or two flagella. The distal half of the cell is much vacuolated and in well-fed specimens these vacuoles contain darkly staining material called by Schneider, 'Sekretballen.' The basal end is darkly granular and bears no myoneme." (Page 521.)

Downing (1905) maintained that the endodermal cells all divide amitotically; but it has been found that these secretory cells divide by mitosis as well as do the endodermal epithelio-muscular cells. The secretory cells have a mitotic figure slightly similar to the endo-interstitial cells from which they arise in that the astral rays, if present, are only very slightly differentiated, and in the apparent absence of centrosomes. Centrioles are found and lie within the nuclear area.

Material and Methods

Pelmatohydra oligactis Pallas was used in the study of mitosis in these secretory cells. *Hydra* having buds, testes, and ovaries were killed in Bouin's, Zenker's, and Carnoy's. Sections were cut at 5 and 7 microns after embedding in paraffin. Staining was done on the slide with iron-hematoxylin and acid fuchsin. Mountings were made in Damar, and the sections were studied with a compound binocular microscope equipped with 97 × oil immersion objective, and 10 × and 15 × eye-pieces.

Prophase

The early stages of the prophase are difficult to follow. The nuclear membrane remains prominent and stains darkly until the spireme breaks up into chromosomes. The nucleolus also remains, apparently unaltered, up until the disappearance of the nuclear membrane. (See Figs. 2 and 3, Plate 1.) The chromosomes are V-shaped and arrange themselves in the equatorial plate with the tips of the V's toward the center of the spindle. The spindle fibers are quite prominent and converge sharply at each pole, which, in many cases, does not touch the surrounding cytoplasm. Astral rays and centrosomes, if present, are not discernible. The centriole up to this stage has not been found, but with the formation of the polar spindle fibers it takes the stain very intensely and lies within the old, slightly enlarged nuclear area.

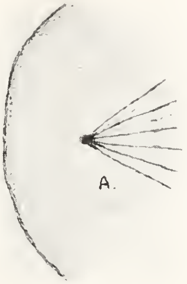

Fig. 1

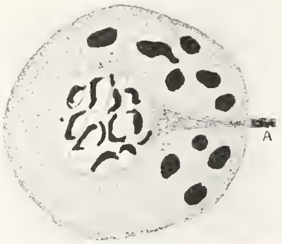

Fig. 2

Text Fig. 1. Section of cell through division center which shows centriole in old nuclear area, *A*.

Text Fig. 2. Daughter cell with remains of spindle fibers at *A;* the centriole being enclosed in nuclear area.

Metaphase

The chromosomes divide longitudinally, division starting at the tips of the V's. As they separate the daughter chromosomes remain connected by interzonal fibers which stain darkly.

Anaphase

As the daughter chromosomes approach the poles their tips fuse upon coming in contact, forming a deeply-staining mass. This mass of chromosomes seems to enlarge slightly when it reaches the area where the new nuclei will be formed. This enlargement is not one of vacuolization, but one of enlargement of the individual chromosome itself prior to vacuolization. The centriole is enclosed within the nuclear area by the formation of the nuclear membrane. (See Text Figs. 1 and 2.)

The interzonal fibers remain until after the cell divides to form the daughter cells. Those portions of it which lie within the area where the new nuclei are to be formed disappear first, leaving a small thread of old interzonal fiber material which remains in the cytoplasm of one of the daughter cells.

TELOPHASE

The polar spindle fibers disappear at the time of the enlargement of the chromosome mass. The chromosomes then fuse and reconstruct the daughter nuclei. The nuclear membranes are re-formed and enclose the centriole within the nuclear area; the nucleolus reappears at about the same time. The old thread of interzonal fibers, which remained in the cytoplasm of one of the daughter cells, disappears at about the time the nucleus reaches its normal size; apparently it is absorbed during the period of growth.

DISCUSSION

Interstitial cells are found both in the endoderm and ectoderm of *Hydra*. As is well known, those of the ectoderm may develop into cnidoblasts, nervous elements, sperm, eggs, and, according to Strelin (1928), they may also be metamorphosed into epithelio-muscular cells

PLATE I

1. Secretory cell from *Pelmatohydra oligactis*. From a preparation fixed in Bouin's and stained with iron-hematoxylin. × 450.

2. Spireme stage of secretory cell with nucleolus still present. Fixed in Bouin's. Stained with iron-hematoxylin and acid fuchsin. × 350.

3. Stage in which the spireme has segmented into chromosomes and the nucleolus has entirely disappeared. Fixed in Carnoy's acetic alcohol and stained with iron-hematoxylin. × 400.

4. Early metaphase seen from the spindle end of the mitotic figure. Fixed in Zenker's. Stained with iron-hematoxylin and acid fuchsin. × 350.

5. Metaphase seen from the side. Fixed in hot Bouin's and stained with iron-hematoxylin. × 350.

6. Late metaphase. Fixed in hot Bouin's. Stained with iron-hematoxylin and acid fuchsin. × 350.

7. Anaphase. Fixed in Zenker's and stained with iron-hematoxylin. × 400.

8. Late anaphase. Fixed in hot Zenker's. Stained with iron-hematoxylin and acid fuchsin. × 350.

9. Telophase. Fixed in Carnoy's acetic alcohol. Stained with iron-hematoxylin and acid fuchsin. × 425.

10. Telophase. Fixed in hot Bouin's and stained with iron-hematoxylin. × 300.

11. Late telophase. Fixed in hot Bouin's and stained with iron-hematoxylin. × 325.

12. Daughter cells. Note old spindle remains in cytoplasm at (*A*). Fixed in Carnoy's acetic alcohol and stained with iron-hematoxylin. × 350.

Plate I

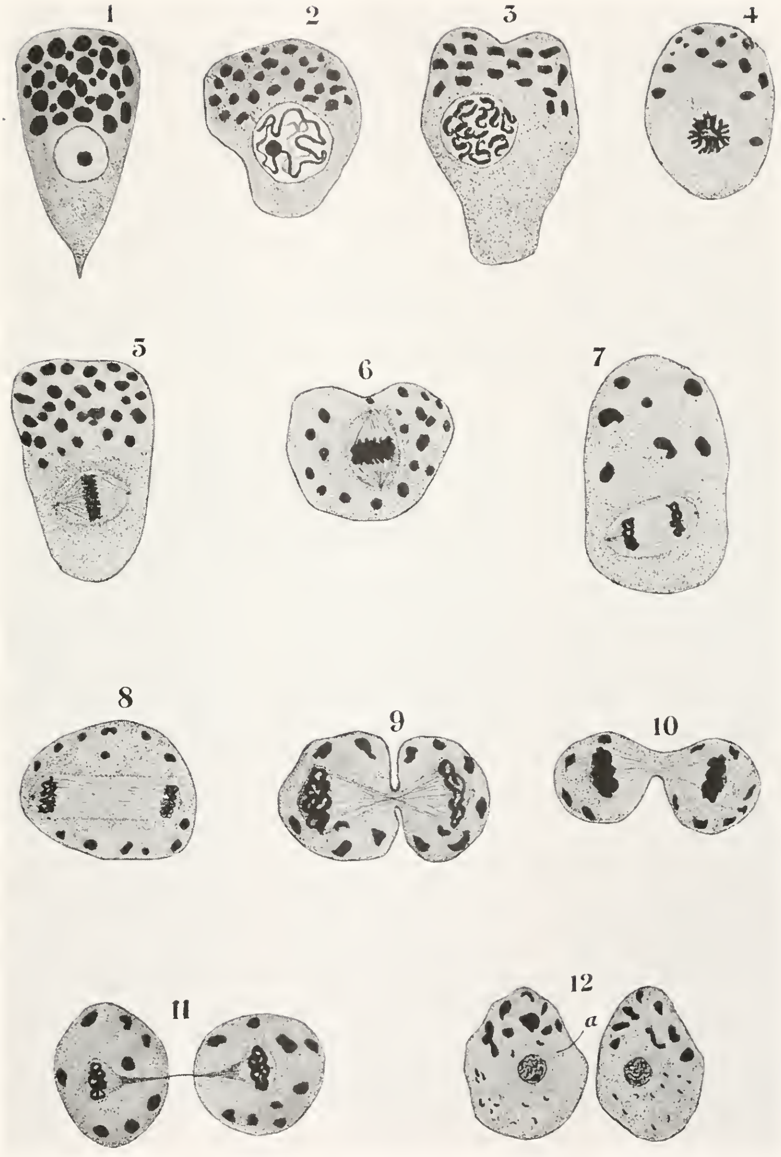
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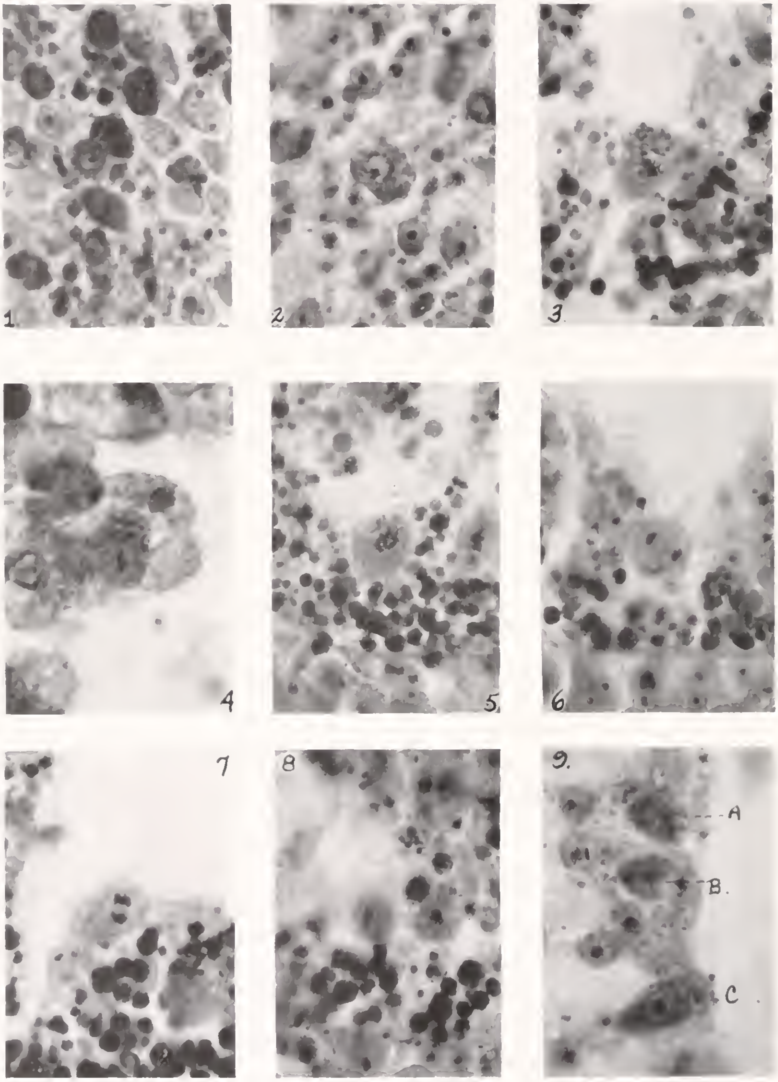
PLATE II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of the ectoderm. The interstitial cells of the endoderm may be elaborated into secretory cells found associated with the epithelio-muscular cells of the lateral walls of the *Hydra's* body. They may also be elaborated into the peristomal-gland cells of the oral region; finally, they may be elaborated into nervous elements associated with the endoderm.

There are, however, several interesting points to be considered in connection with the interstitial cells relative to their location in the *Hydra's* body. Downing (1905), in his work on sperm formation, has shown that those interstitial cells of the ectoderm which are taking part in sperm formation go through a series of mitotic divisions. Again, in his work on ovogenesis (1909) he has shown that the egg arises from an interstitial cell of the ectoderm and, at maturation, casts off two polar bodies. These observations were confined to the interstitial cells which were involved in the sexual activity of the *Hydra;* however, when one studies the interstitial cells of the ectoderm which are developing into cnidoblasts he is forced to the conclusion that after the anlage for a nematocyst has appeared in the cytoplasm of the ectodermal interstitial cell, that cell is incapable of further division.

When one studies the endodermal interstitial cells he finds that they are able to divide by mitosis. When he further studies the cells of the endoderm which arise from these endodermal interstitial cells, he finds that they too are able to increase their number by mitosis after they have been metamorphosed into secretory or peristomal-gland cells; as well as after the secretory particles, which are functionally of a different nature in these two groups of cells, have appeared in their cytoplasm. This is quite in contrast to some of the ectodermal interstitial cells, which, after once being destined to play a part in the general activity of the *Hydra's* life, are incapable of further division.

PLATE II

1. Section through general endoderm to show distribution of the secretory cells. Bouin's; iron-hematoxylin and acid fuchsin. × 776.
2. Early prophase.—Bouin's; iron-hematoxylin and acid fuchsin. × 776.
3. Prophase of a secretory cell which contains already formed secretion granules. Bouin's; iron-hematoxylin and acid fuchsin. × 776.
4. Metaphase of a secretory cell containing secretory granules. Bouin's; iron-hematoxylin and acid fuchsin. × 776.
5. Metaphase seen from the end. Bouin's; iron-hematoxylin and acid fuchsin. × 776.
6. Anaphase. Carnoy's acetic alcohol; iron-hematoxylin. × 776.
7. Anaphase. Carnoy's acetic alcohol; iron-hematoxylin and acid fuchsin. × 776.
8. Late telophase. Carnoy's acetic alcohol; iron-hematoxylin and acid fuchsin. × 776.
9. Daughter cells at *A* and *B*. Compare with normal secretory cell at *G*. Bouin's; iron-hematoxylin and acid fuchsin. × 776.

SUMMARY

1. The secretory cells are able to divide by mitosis.

2. The method of mitosis is similar to that of the endodermal interstitial cells from which they arise.

3. This mitosis may take place after the secretory granules have appeared in the cytoplasm of these cells.

4. The secretory cells are able to increase their number after being destined to play a part in the activity of the *Hydra's* body; in contrast with some of the ectodermal interstitial cells which, after being destined to play a part in the activity of the *Hydra*, are incapable of further increasing their number.

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THE GROWTH OF THE NUCLEUS IN THE DEVELOPING EGG OF CHLOROHYDRA VIRIDISSIMI

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Introduction and Historical

The development of the egg of *Hydra* has been studied quite thoroughly by many workers, chief of whom are Kleinenberg (1872), Brauer (1891), Downing (1908), Tannreuther (1908), Wager (1909), and Kepner and Looper (1926). All of these workers have pointed out more or less definitely that the eggs arise from indifferent cells, that the developing egg grows at the expense of the surrounding modified interstitial cells which are eventually taken into the egg itself, and that apparently only one nucleus survives and becomes the nucleus of the egg. It is interesting to note in passing that Kepner and Looper (1926) were the first to show that the earlier stages of the development of the egg are more dependent upon the endoderm for their substance than upon the modified interstitial cells of the ectoderm surrounding them. This fact had apparently been overlooked by other workers along this line. Most workers have come to the conclusion that the egg arises from an apparently unmodified, interstitial cell of rather average size, which in its earlier stages cannot be distinguished from the other interstitial cells until the process of ovary formation is very much advanced. Several workers have observed that from one to several eggs may start simultaneous development in an ovary. Usually, however, only one survives and grows to maturity. Downing (1909), on the other hand, found that an ovary of *H. diœcia* may produce several eggs, and further, advances the theory that the interstitial cells which will later develop into eggs are always distinguishable from the other interstitial cells by their size, peculiar nucleus and cytoplasmic inclusion adjacent to the nucleus, and that they are self-propagating and thus handed down from generation to generation.

These studies and differences of opinion, however, deal mostly with the origin of the ovary, the development of the eggs by taking their substance from the endoderm during the earlier stages, and the appropriation of the surrounding modified interstitial cells during the later stages of growth; but since none of these works deal with or demonstrate, except in a minor way, the development of the nucleus of the egg, it was decided to make a study of it at its various periods of development.

MATERIAL AND METHODS

Chlorohydra viridissimi was used exclusively in this experiment. A well-balanced laboratory culture was made early in the month of August, and the animals became sexually mature about September first. It was observed that these *Hydra* were producing only one ovary from which apparently only one egg was being matured, so they thus lent themselves quite admirably as material for the study of the development of the nucleus. The method of studying these nuclei was as follows: The *Hydra* were removed to a slide upon which a square of vaseline had been marked out with a match stick. The *Hydra* were placed in the center of this square in as large an amount of Mundie's macerating fluid as possible and then covered with a cover-glass. By drumming very lightly on the cover-glass with a needle it was possible to work the egg out of the ovary; further slight drumming burst the egg and allowed the nucleus to be seen. By carefully sliding the cover-glass to one side or the other it was possible to roll the nucleus out and away from the majority of the surrounding pseudocells where it could be studied, measured, and photographed. Extreme caution was exercised to see that the nuclei were not brought under any pressure so that they remained in their original round condition. When these nuclei were measured, care was taken that the focusing was so adjusted that the nuclei were measured at their equator; this same precaution was exercised when they were photographed. In addition to the above, a series of measurements and observations were made upon nuclei which had been worked out in aquarium water. As far as could be ascertained no measurable shrinkage or swelling could be found, so the work was continued using the Mundie's macerating fluid since the nuclei could be preserved in their almost original condition for from 15 to 20 hours, if precautions were taken to see that the cover-glasses were well sealed with vaseline.

A compound binocular microscope, equipped with 10 ×, 43 ×, and 97 × objectives, and 5 ×, 10 ×, and 15 × oculars, was used in this study.

THE INTERSTITIAL CELLS

Leydig (1848) was the first worker to describe the interstitial cells of *Hydra*. These small cells lie between the epithelio-muscular cells of both the ectoderm and endoderm. They are most numerous in the ectoderm between the epithelio-muscular cells of the upper two-thirds of the *Hydra*'s body; one rarely finds undifferentiated interstitial cells on or in the tentacles; in the stalk and basal disc they are also often en-

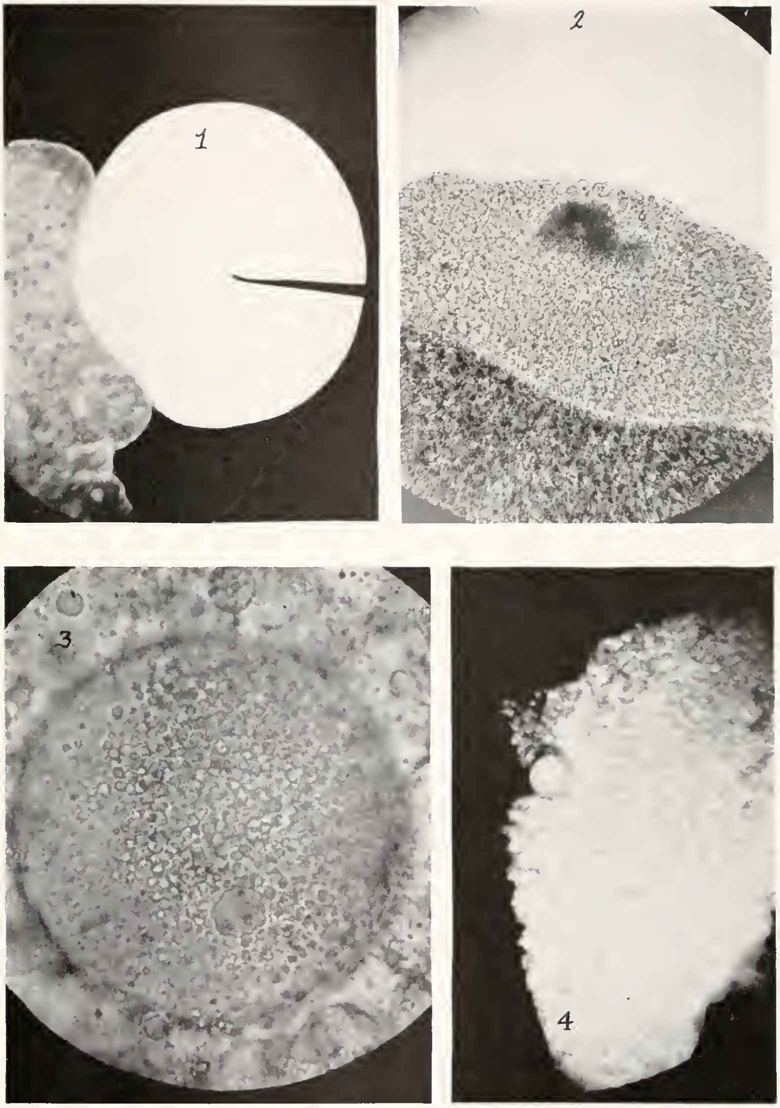

Plate I

1. Mature egg before process of maturation has set in. Tip of pointer indicates position of nucleus. × 90. Dark field illumination photograph.

2. Dark area indicates a very small egg in the ovary. × 90.

3. Mature nucleus just before process of maturation has set in. × 776.

4. Dark field illumination photograph of an egg crushed in Mundie's macerating fluid. Note size of nucleus as compared to the surrounding pseudocells. × 90.

countered in varying numbers. Various workers have slightly different terminologies in describing these cells, such as follows:

First: Those cells which were developed or were developing into stinging cells have been called "cnidoblasts," "cnidocyts," "nematoblasts," and "nematocyts"; some have even used the word "nematocyst" to designate the interstitial cell with its nucleus, cytoplasm, and cell organelle, or coiled stinging thread.

Second: Some have called all of the cells which lie between the epithelio-muscular cells "interstitial cells" regardless of whether they were in any wise differentiated and,

Third: Some have used the words "indifferent cells" to designate those which were in no wise differentiated. We see then that some ambiguity has arisen through the various terminologies which have been employed. It is true, they are all "interstitial cells," but not all are "indifferent cells." The interstitial cells include all of the cells which lie between the epithelio-muscular cells of the body of the *Hydra* of both the endoderm and the ectoderm. The "indifferent cells," which are also interstitial, are the interstitial cells of both the endoderm and the ectoderm which are not differentiated in any way and which may be modified as needed to various specialized interstitial cells of either endoderm or ectoderm. The total number of "interstitial cells" remains almost constant in relation to the size of the animal; but the number of "indifferent cells" apparently varies greatly at different times; these cells also vary greatly in size and location; sometimes they lie down close to the bases of the epithelio-muscular cells near the mesoglea, at the same time others are found out near the inner surfaces of the epithelium; they may also be found among groups of developing cnidoblasts or in great groups alone. They are occasionally found, apparently unmodified, in developing ovaries and under testes. Their shape varies

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PLATE II

1. Light field illumination photograph of an egg crushed in Mundie's macerating fluid. × 90.

2. The nucleus after maturation as it awaits fertilization at the bottom of a small depression in the egg membrane. Compare with No. 6, Plate III. × 776.

3. Nucleus of a very young egg. Note nucleolus. This was the smallest nucleus found in macerated material. Nuclear diameter 17.5 microns. × 320.

4. From macerated material. Nuclear diameter 24.5 microns. × 320.

5. Macerated material. Nuclear diameter 41.5 microns. × 320.

6. Macerated material. Nuclear diameter 43.80 microns. × 320.

7. Macerated material. Nuclear diameter 54.25 microns. × 320.

8. Macerated material. Nuclear diameter 63.5 microns. × 320.

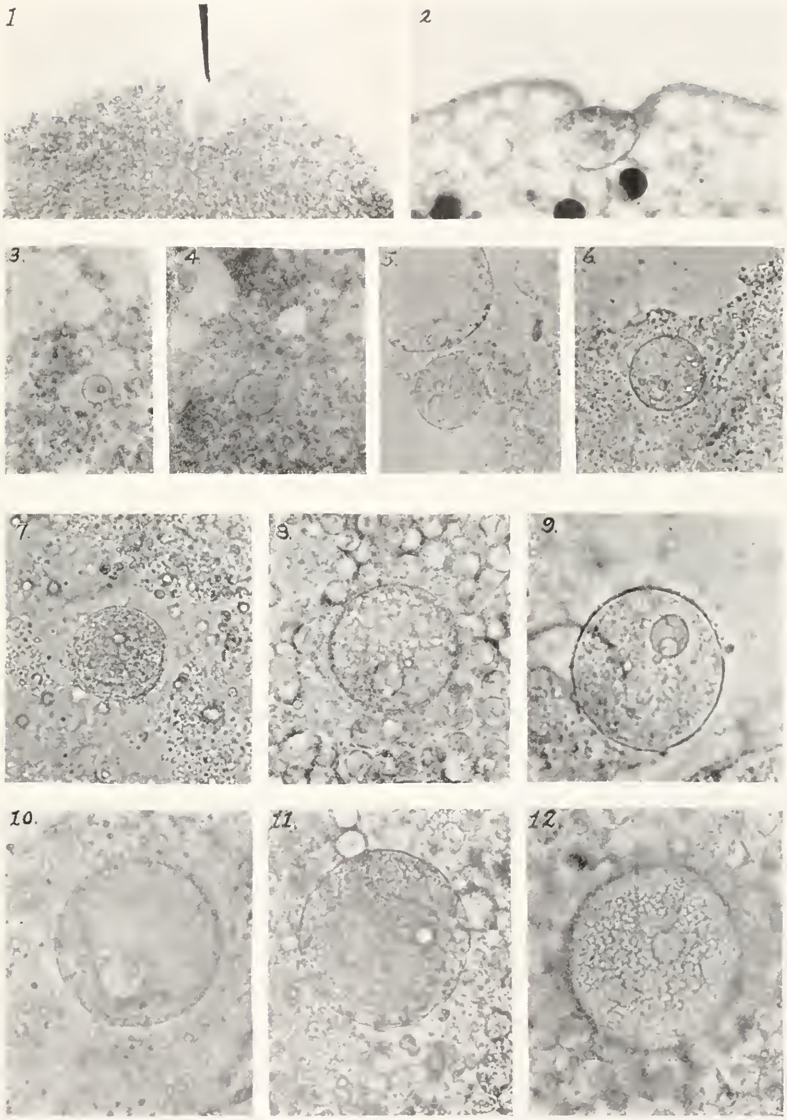
9. Macerated material. Nuclear diameter 86.75 microns. × 320.

10. Macerated material. Nuclear diameter 94.5 microns. × 320.

11. Macerated material. Nuclear diameter 96.25 microns. × 320.

12. Macerated material. Nuclear diameter 103 microns. × 320.

Plate II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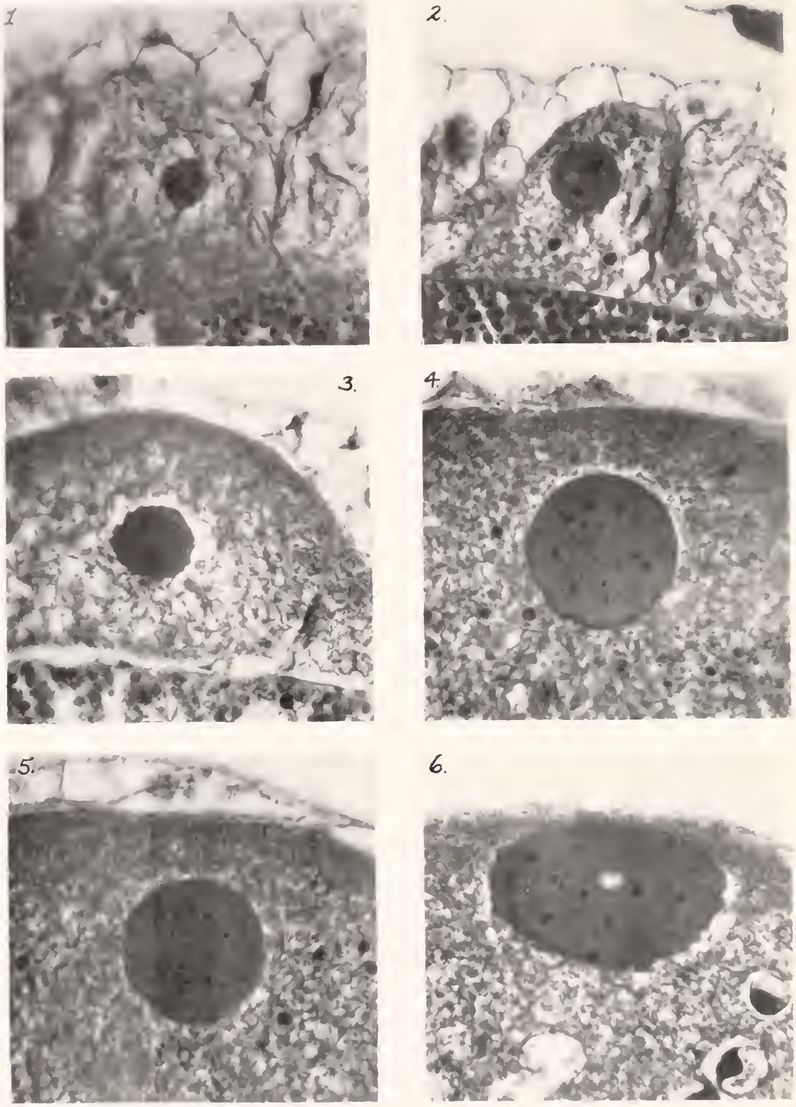


PLATE III

1. Fixed material. Nuclear diameter 9.25 microns. × 450.
2. Fixed material. Nuclear diameter 12.95 microns. × 450.
3. Fixed material. Nuclear diameter 20.50 microns. × 450.
4. Fixed material. Nuclear diameter 38.50 microns. Note how the nucleoli have been increased in 4, 5, and 6. × 450.
5. Fixed material. Nuclear diameter 37.50 microns. × 450.
6. Fixed material. Nuclear diameter 110 × 90 microns. Note that this nucleus is lying just under the egg membrane. × 450.

greatly in accordance with the number of other interstitial cells in the neighborhood and the region of the body in which they are found. The expansion and contraction of the animal's body, it has also been noted, vary the shape of the cells somewhat. During the late prophase of mitosis they assume a rounded condition. The nucleus has a very distinct membrane, and the entire nucleus takes the commoner nuclear stains quite readily and quite intensely. The number of nucleoli varies from one to three, and they vary greatly in their ability to take up various staining solutions.

The Appearance of the Egg and Its Later Development

In sectioned material the egg may be identified much earlier than in macerated material. As has already been pointed out in many works along this line, one of the primary steps of ovary formation is the increase in size of the indifferent cells in the area where the ovary is to be formed. This increase in size continues, in many cases, until these cells have enlarged from eight to ten times their original size. During the process of enlarging, their cytoplasm becomes highly vacuolated and their nuclei degenerated. In studying sections through an ovary one finds that the future eggs do not present any of the characteristics of the modified indifferent cells and take the ordinary stains quite differently from the surrounding cells which they later engulf. When the *Hydra* bearing ovaries are studied slightly compressed the eggs may be identified at an early stage by their color, position in the ovary, and different refraction of the light passing through the ovary. However, it is very difficult to identify the eggs in this manner until they are larger than the average modified indifferent cells which surround them.

Kepner and Looper (1926) have shown that the egg depends first upon the endoderm for its material. This process, as they have demonstrated, is continued until the egg is of quite large size and has developed many pseudopodia which radiate from a more or less central area in all directions between the cells of the ectoderm. Many observations, made on both sectioned and living material, have convinced me that these pseudopodia never invade a region or area in which the indifferent cells are not modified. The boundaries of the ovary are very distinct during all of the process of ovogenesis.

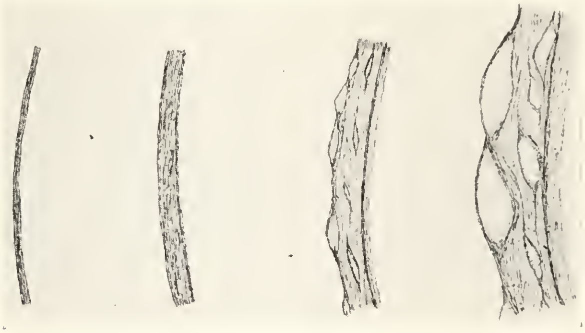
The nucleus grows very fast and apparently is always in proportionate size to the size of the egg. It is very difficult to remove entire eggs from the ovary at an early stage of their development, but during their later stages they may be removed entire if caution is taken. In this case they are not worked out in Mundie's macerating fluid but in the water from the aquarium. A series of measurements of the eggs and their nuclei is given below in Table I. These measurements were made on eggs and nuclei just before the process of maturation. We see from this table then that the average relation of diameter size is 1 to 9.66 + for the egg just before the process of maturation took place. Whether this nucleo-cytoplasmic relation has been maintained through the various periods of development is rather difficult to say, but it is interesting to note that several authors have observed that some eggs in their earlier stages are possessed of several nuclei, only one of which survives. Would this indicate then that this cell, which is to be the future egg, can increase in size only in relation to one nucleus or to only a given amount of nuclear material? If we would describe briefly the various stages of development from indifferent cell to the egg as it awaits fertilization, we should see that we have:

TABLE I

	Egg Diameter *microns*		Nuclear Diameter *microns*
1	1022.17		104.25
2	1035.50		103.5
3	1043.50		101.35
4	1124.2		115.45
5	1145.26		116.49
6	1178.47		116.78
7	1190.55		112.46
8	1232.48		134.19
9	1374.36		146.23
10	1413.57		156.42
Av. diameter egg	1176.004	Av. diameter nucleus	121.712

1. Indifferent cell (deriving liquid material from endoderm).
2. Very much enlarged indifferent cell (deriving liquid material from endoderm).
3. Very actively amœboid young egg (deriving solid and liquid material from endoderm and ectodermal ovarian cells).
4. The egg ceases amœboid activity and rounds up; nucleus lies near center of egg mass.
5. Migration of nucleus to periphery of egg.
6. Formation of first and second polar bodies with a consequent reduction of nuclear size to about one-eighth of its size at Stage 4.

We see then that this nucleus, up to Stage 4, is controlling a certain amount of cytoplasm; or vice versa, that the cytoplasm is increasing in amount in relation to a certain proportion of nuclear material. It appears that the cessation of the taking in of more material would indicate that after the nucleus has reached a certain size it controls the action of the cytoplasm, since it has been observed many times that when this amœboid activity ceases and the rounding-up process takes place there are many pseudocells very opportunely placed so that the cytoplasm of the egg could take them in with, may I be permitted to say, very little effort. Would this not indicate that the nucleus is through with its growth, since it allows no more additions to the cytoplasm, and that, at least at this period, it has a definite nucleo-cytoplasmic relation? This growth (to a certain size in relation to the cytoplasm) having been reached, the nucleus goes into the process of maturation during which the nuclear material is reduced. During this process the cytoplasm remains comparatively dormant and does not take any part in the cell's activity until the nucleo-cytoplasmic relation has been restored by the entrance of the sperm, which again restores the relation that previously existed.



TEXT FIG. Nuclear membrane from normal to maturation stage. × 847.

THE NATURE OF THE GROWING NUCLEUS

By the methods employed in this study, one is unable to see the small dark ovoid body near the nucleus or to measure the relation of the nucleus to the cytoplasm with any accuracy except in the later stages. The nucleus of the young egg, however, can always be distinguished from the nuclei of the surrounding enlarged interstitial cells. This distinction lies in its different refraction of the light and its more solid appearance. The nucleoli vary greatly in number during the growth of the nucleus and are apparently produced by the fusion of the many small nucleoli which are constantly being formed. The larger nucleolus apparently grows also in proportion to a certain nuclear size. One is constantly impressed, while observing these nuclei, that as far as appearance is concerned from the earliest stages up to the last stages no special change takes place except that of enlargement. The nuclear membrane, which seems to be thicker and more durable than that of other indifferent cells, is quite evident during the entire process of growth. The nodal points of the net enlarge; this growth seems to move inward toward the center of the nucleus during the entire process of growth. At the period of cessation of growth these enlarged nodal points give to the nucleus the appearance of being filled with thousands of large granules all very tightly packed together. The nuclear membrane at this stage is very tough and thin, and one soon learns to distinguish the nuclei which have just completed their growth by this characteristic. Later, this membrane is the first part of the nucleus to show evidences of change incident to the earlier stages of maturation as it increases in thickness and takes on a rather spongy appearance (see text figure). The nuclei which are in the earlier stages of maturation and display membranes such as the above were observed to shrink in size and eventually to become very much wrinkled over their surfaces as if the contained liquids of the nucleus were passing through this membrane into the surrounding medium. Nuclei which displayed the thin tough membranes were observed to maintain the same size over long periods of time, one having been kept for a period of thirty-eight hours.

SUMMARY

1. The nucleus apparently maintains a relation of 1 to 9.66 to the cytoplasm in the development of the eggs of *Chlorohydra viridissimi.*

2. It appears that when the nucleus has attained a certain size no more cytoplasm is added to the egg.

3. During the process of growth of the nucleus the nodal points of the net increase greatly in size.

4. The number of nucleoli increases in relation to the size of the nucleus.

5. The nuclear membrane becomes highly vacuolated just prior to the process of maturation.

6. During the process of maturation the nuclear volume of the egg is reduced to about one-eighth of its volume prior to maturation.

ACKNOWLEDGMENTS

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ION EFFECTS UPON ION PERMEABILITY OF THE *FUNDULUS* CHORION

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I

An electrical potential difference arising between two electrolyte solutions which differ in either concentration or composition is pretty generally interpreted to mean that the net conveyance of positive and of negative ions across the boundary between them is unequal. This is true for the case when the two solutions are free to diffuse into one another. It is also true when they are separated by a membrane which modifies the speeds of ionic diffusion. It is a principle, moreover, equally conceded by those who attribute to the boundary membrane a porous structure permeated by water channels, and by those who assume it to be a strictly non-aqueous phase. Even if water transport across the membrane is occurring simultaneously with ionic diffusion, the magnitude and direction of the observed potential difference must still be a function of the net transport of positive and of negative ions in the two directions. A difference in concentration of positive and of negative ions is admittedly incapable of demonstration by chemical analysis, but this is to be expected; the opposing force of electrostatic attraction must confine the difference to exceedingly minute layers of solution. Absolute rates of ion movement can not be gauged by a measurement of potential difference. But, as a criterion for differential ion transfer, electrical polarity across the boundary between the two electrolyte solutions seems to be remarkably unambiguous.

This criterion has been used in the study of a variety of membranes. In the case of the chorion of single *Fundulus* eggs it seemed to show that at ordinary reactions that membrane is relatively permeable for cations, relatively impermeable for anions (Sumwalt, 1929), a result which satisfactorily explains the long-standing observation of Loeb and Cattell (1915) that K escapes more easily from the egg into a solution of some electrolyte than into distilled water. The same study showed that when the H ion concentration is sufficiently increased, the chorion becomes more permeable for anions than for cations, and that this reversal of preferential ion permeability occurs in KCl solutions at about pH 3.7. It was suggested, however, on the basis of a very limited number of experiments with two other salts, that the H ion concentration critical for reversal might vary according to the salt in which the membrane potentials are measured.

This suggestion, made originally on the basis of an experimental cue, fits theoretically into a conception of membrane potentials as fundamentally related to diffusion potentials. A study of the reversal point in several salts could therefore furnish evidence for or against the theory that membrane potentials are in some sense modified diffusion potentials.

The problem has also a slightly more practical side. *Fundulus* eggs have been subjected to certain experiments by Loeb (1912–1922) and by Armstrong (1928), which showed a salt antagonism, greater or less according to the salt used, for acid penetration into the egg. Armstrong has shown satisfactorily that this effect takes place chiefly at the ectodermal surface of the embryo. He has not shown, however, that it does not also occur at the surface of the chorion. If a dependence of the ion permeability of the *Fundulus* chorion upon the salt content of the medium, as well as upon the pH, could be demonstrated, it might by analogy furnish an interesting step in explaining the mechanism of salt antagonism for acid penetration through the ectoderm of *Fundulus* embryos.

II

With these two objects in mind, the earlier experiments of Sumwalt on potential differences across the *Fundulus* chorion have been extended to compare three salts, $CaCl_2$, KCl, and K_2SO_4, throughout a range of pH values from 2.0 to 6.0. The particular criterion chosen for this study of ion permeability was the way in which electrical polarity across the membrane was affected by varying the concentration, rather than the composition, of the solutions applied to the membrane. Therefore, the potential differences observed are termed "concentration potentials". Measurements were made in the manner described in detail in the earlier paper. In brief, one measurement of concentration potential in KCl consisted of the following procedures: The chorion of an egg was impaled on the capillary point of a saturated KCl calomel electrode, while the other similar electrode was dipped into the sea water in which the egg was immersed. The sea water was drawn off, KCl M/20 of the desired pH substituted, and a measurement of potential made by means of a simple potentiometric circuit and a sensitive galvanometer used as a null instrument. KCl M/20 was then replaced by KCl M/200 at the same pH, and another measurement made. With this dilute solution, several fresh washings were necessary before the potential attained a steady value. The algebraic difference between the values in the dilute and concentrated solutions is the concentration potential.

It will be observed from this synopsis that the two solutions of differing concentration were applied successively to the outside of the chorion. It is assumed that the potential difference obtained in this way is equal to that which would be measured if both solutions could have been simultaneously applied outside and inside of the membrane.

Solutions were adjusted to the desired pH values by addition of that acid or base which would add no new ion to the mixture (*e.g.*, KOH or HCl to KCl). A difference of not more than 0.1 of a pH unit, and usually of less than 0.05, was tolerated between the dilute and concentrated members of any given pair of solutions used in a measurement. The necessary pH determinations were made with a quinhydrone electrode calibrated against standard buffer solutions. Those solutions described as having a pH of 5.0 were made up in unboiled distilled water without the addition of any acid or alkali. A few quinhydrone electrode determinations seemed to show that their pH was somewhere between 5.0 and 6.0, as might have been predicted; but determinations of the pH of such solutions are of doubtful meaning, and the exact value of the pH of these more alkaline solutions is not important for the shape of the curves obtained nor for the conclusions drawn from them.

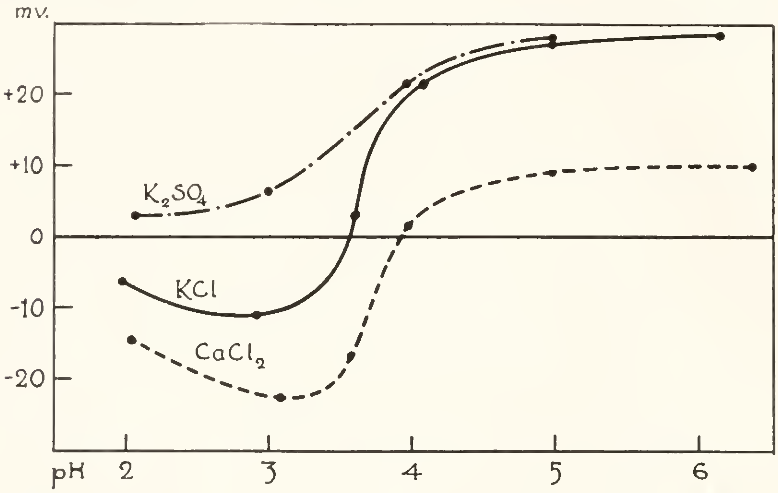

FIG. 1. Concentration potentials across the chorion between M/20 and M/200 solutions at various pH values. The pH plotted is that of the dilute solution, but the pH of the concentrated solution differed from this by less than 0.1. Each point represents an average of values obtained on three different eggs. See Table I.

Concentration potentials measured between M/20 and M/200 solutions of KCl at pH 5.0 were of the order of magnitude of 28 millivolts and of such polarity that the dilute solution was positive relative to the concentrated (Fig. 1). In acid solutions, at pH values of 2.0 and 3.0, the dilute solution was relatively negative, and the concentration potential somewhat smaller than in "neutral" solutions. The reversal of membrane polarity occurred along a smooth curve which crossed the line of zero potential at about pH 3.5.

In K_2SO_4 solutions the concentration potentials at pH 4.0 and above were virtually those obtained also with KCl. As acidity increased, the K_2SO_4 curve diverged from KCl, descending with a much more gradual slope toward the zero potential line, and not crossing it at any of the reactions studied in the set of experiments shown (though small reversed concentration potentials at the most acid pH of 2.0 did occur in a few experiments with another lot of eggs).

On the other hand, in $CaCl_2$ solutions, acidity brought about a reversal of concentration potential more readily than in KCl. In the acid-free solutions of $CaCl_2$, concentration potentials were somewhat less than half of those yielded by KCl or K_2SO_4. Reversal of membrane polarity occurred at about pH 3.9, and in acid solutions the concentration potentials were approximately twice those observed with KCl at corresponding reactions.

These experiments were performed on developing eggs ten days old; they had been kept at a temperature of 11° C., however, since their fifth day, so that their development corresponded approximately to that of seven-day eggs. In order to evade difficulties engendered by the variability of *Fundulus* eggs from different batches, the data presented in Fig. 1 were secured within thirty hours on eggs from a single female. Each point in the graph represents an average of concentration potentials from three different eggs. A table of the data from which Fig. 1 was drawn is given to show the small spread of the individual measurements (Table I). Additional preliminary experiments performed on various batches of eggs gave values which differed from these in some respects quantitatively, but not in any qualitative sense. Therefore, although no great significance can be attached to the exact pH value at which the reversals occur, the directional effects of varying degrees of acidity and the comparative influence of the three salts are reasonably certain.

The results, interpreted according to the principle invoked at the beginning of this paper, constitute an answer to the more practical object of this study. They signify that in acid-free solutions the *Fundulus* chorion is more freely permeable for K^+ than for $SO_4^=$ or Cl^-, and more permeable for Ca^{++} than for Cl^-, whereas in solutions of considerable acidity it becomes more permeable for Cl^- than for any other ion of the salts studied. Furthermore, the tendency of acid to increase the relative permeability of the chorion for anions appears to be augmented by the presence of Ca^{++}, but antagonized by the presence of $SO_4^=$, as compared with an arbitrarily chosen standard behavior in KCl solutions.

Table I

Concentration potentials across the chorion measured between M/20 and M/200 solutions. The sign of the dilute solution was positive except where the negative sign occurs. The pH indicated is that of the more dilute solution, from which the concentrated solution differed by not more than 0.1 of a pH unit. Fig. 1 is drawn from these data.

KCl			K_2SO_4			$CaCl_2$		
pH	Concentration Potential	Av.	pH	Concentration Potential	Av.	pH	Concentration Potential	Av.
	mv.	*mv.*		*mv.*	*mv.*		*mv.*	*mv.*
1.98	9.0 − 4.4 − 4.9 −	6.1 −	2.07	3.4 4.4 1.2	3.0	2.04	16.2 − 14.5 − 12.3 −	14.3 −
2.92	7.5 − 13.2 − 12.1 −	10.9 −	3.07	5.0 6.1 9.1	6.7	3.08	24.2 − 23.7 − 19.8 −	22.6 −
3.60	7.2 0.7 1.3	3.1				3.57	17.3 − 15.5 − 16.2 −	16.3 −
4.09	22.0 20.4 21.8	21.4	3.96	19.9 21.1 21.4	20.8	3.98	3.1 1.7 0.2	1.7
5.	28.3 27.5 25.2	27.0	5.	27.1 28.5 28.2	27.9	5.	10.3 8.7 7.7	8.9
6.17	31.1 30.3 23.2	28.2				6.40	6.8 8.8 14.2	9.9

Certain salts differ from one another in their effectiveness as antagonists of acid penetration into *Fundulus* eggs according to the experiments of Loeb (1912–1922), and of Armstrong (1928). The conclusions just described concerning the permeability of the chorion for ions may be brought into relation with those experiments by the following crude but suggestive theoretical chain. Acid penetration must depend not only on the concentration and speed of the H ion in traversing the membrane, but also upon the concentration and penetrating power of the acid anion outside (or of some cation within the egg capable of exchange for H ions). Hence, increasing acidity in the medium around the egg might favor acid penetration, not only for the primary and obvious reason that it increases the concentrations of the H ion and of the acid anion; but also for the additional reason that it possibly increases the permeability of the membrane for anions. For instance, if the negative charges borne on a membrane were a hindrance to anion penetration, abolition of these charges would bring about increased penetration of the negative ions. Whether relative increase of permeability for anions signifies an absolute increase of permeability for anions is not revealed by measurements of electromotive force alone, but there is no evidence against such a possibility. This secondary influence of acidity upon acid penetration, then, is the factor which the nature of the salt present could influence, if the salt tends to combine with the proteins of the membrane. A divalent positive ion like Ca may be supposed to be more potent than K in effecting combination with the membrane and so neutralizing its negative charges and bringing it nearer to its isoelectric point. To be specific, acid might be expected, on the basis of the results here recorded, to penetrate more

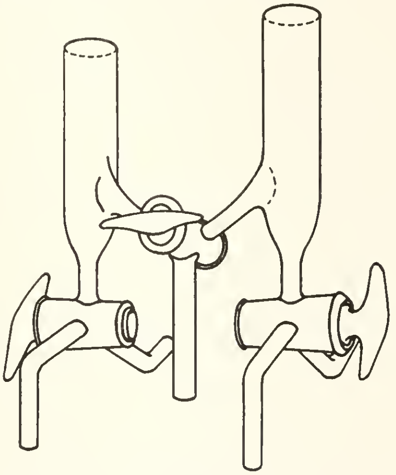

FIG. 2. The apparatus of Dr. Kunitz for measurements of free diffusion potentials. A saturated solution of KCl fills the rear tubes which are to be connected with calomel half cells. In use, the two reservoirs are stoppered so that a difference in level of the two solutions can not cause mixing. If a flowing junction is desired, air may be admitted slowly through these stoppers under the control of screw clips.

readily in the presence of $CaCl_2$ than with KCl, and more readily with KCl than with K_2SO_4. It is impossible to make a more detailed application of this suggestion to the work of Loeb and of Armstrong, because neither ever used simultaneously the three salts with which the present experiments have been concerned and because, as has been mentioned already, their experiments dealt with the surface of the embryo rather than with the chorion. The present results must therefore be of interest in a general way, suggestive of a mechanism which might exist at any membrane of structure essentially analogous to that of the chorion. This explanation of the results is purely speculative. But the results themselves, stated in terms of relative ion permeabilities, depend only on the semi-axiomatic premise of the introductory paragraph.

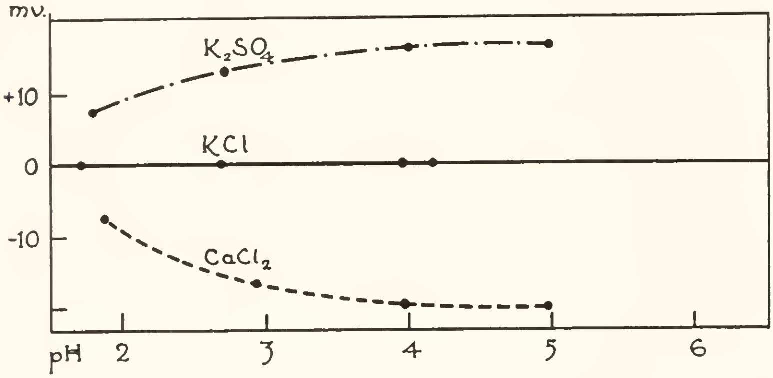

FIG. 3. Concentration potentials across a flowing liquid junction between M/20 and M/200 solutions at different pH values. These results were obtained with the apparatus shown in Fig. 2.

III

To pursue the more fundamental question of the rôle played by diffusion in the origin of membrane concentration potentials, measurements were made of free diffusion potentials between M/20 and M/200 solutions similar to those used in the study of the chorion. They were placed in contact with one another at a flowing liquid junction, and contacts, as before, were saturated KCl calomel electrodes.[1] The results are shown in Fig. 3. Throughout the pH range studied, no potential difference appeared between solutions of KCl. With acid-free K_2SO_4, the dilute solution was positive to the concentrated by 16.6 millivolts, an amount which diminished gradually along the descending pH scale to a value of 7.5 millivolts at a pH of 1.8. The dilute solution of $CaCl_2$, on the other hand, was negative to the concentrated by 20 millivolts, a potential difference which likewise continuously decreased with increasing acidity, to reach a value of 7.5 millivolts at pH 1.9.

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[1] I was enabled by the kindness of Dr. Kunitz to use an apparatus which he had designed and used for similar measurements in connection with Loeb's work on anomalous osmosis. A description of it has not been published. It is therefore shown in Fig. 2. The peculiar advantage of this apparatus lies in its prevention of the mixing of the two solutions due to a difference in level in the two cells.

The diffusion potentials which might have been expected between the solutions used were calculated by the Henderson equation for liquid junction potentials as given by Michaelis (1926, page 179) and are shown in Table II, together with values at corresponding H ion concentrations interpolated on the experimental curves, for comparison. Experimental and calculated values agree very closely in KCl and $CaCl_2$ solutions. In K_2SO_4 an unaccountable systematic discrepancy appears.

Table II

Liquid junction potentials between M/20 and M/200 solutions, (*a*) calculated by the Henderson equation, (*b*) obtained by interpolation on experimental curves. The sign of the dilute solution was positive except where the negative sign occurs.

pH	KCl		K_2SO_4		$CaCl_2$	
	Calc.	Exper.	Calc.	Exper.	Calc.	Exper.
	mv.	*mv.*	*mv.*	*mv.*	*mv.*	*mv.*
2.0	0.11 –	0.0	7.0	9.0	9.7 –	8.8 –
3.0	0.27 –	0.0	12.3	14.5	17.6 –	17.0 –
4.0	0.33 –	0.0	13.3	16.4	19.5 –	19.8 –
5.0	0.33 –	0.0	13.4	16.6	19.7 –	20.0 –

Comparative values for concentration potentials across the chorion and across a liquid boundary being now at hand, the question as to what extent these membrane potentials are modified diffusion potentials may be approached. There are two dominant points to emphasize. The first is that when the membrane potentials are " corrected " by the subtraction of the diffusion potentials, the curves for the three salts nearly coincide at the pH value where zero concentration effects are obtained (Fig. 4). This suggests that the membrane is characterized by an isoelectric point, a pH value where the dissociation of acid and basic groups is equal; and that at this pH the membrane influence upon ionic diffusion is withdrawn, leaving ions free to traverse it at the relative rates characteristic of them in free diffusion. The second important point is that at other pH values the membrane exerts an influence which favors cations in more alkaline solutions, anions in those more acid. The results favor the theory that diffusion potentials do indeed play some part in determining the magnitude and sign of membrane potentials as ordinarily measured, and that at one particular pH value they are the only important factor.

Two minor features of the "corrected" curves (Fig. 4) require brief comment. First, the values of the potentials at pH 2.0 and 3.0 are considerably smaller than at pH 4.0 and 5.0, whereas curves might have been casually expected which were roughly symmetrical with respect to the reversal point. This may possibly be due in part to some obscure electrical property of the membrane. It is certainly partly due to the fact that as the H ion concentration becomes a significant part of the total ion content, the ratio of total electrolyte concentrations on the two sides, upon which potential difference depends, is somewhat reduced. Free diffusion potentials show a similar diminution with increasing acidity, for a like reason. Finally, it seems quite probable that a destructive effect of the acid may partly abolish the differential ion permeability of the membrane before a measurement of concentration potential can be completed. As was mentioned in the earlier paper, the effects of acid upon the chorion even at pH 3.0 were not reversible.

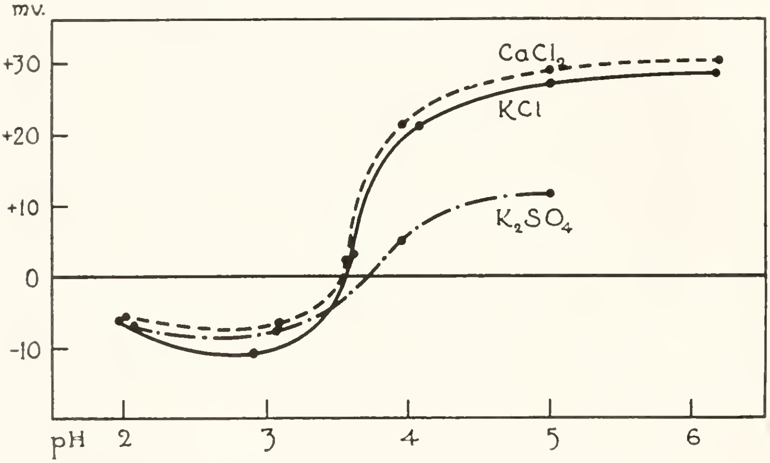

FIG. 4. Membrane potentials minus liquid junction potentials. Such "corrected" membrane potentials probably represent the membrane influence upon concentration effects, more or less freed from the contribution which free diffusion potentials make.

Second, attention must be called to the fact that certain effects of valence upon the magnitude of concentration potentials across the chorion, predicted and found in the earlier study of the *Fundulus* egg, are found again in the experiments here reported; but they are abolished or inverted when the correction of subtracting diffusion potentials is introduced. Not even a suggestion can be offered, however, in the light of our present knowledge, to explain this relation between valence and "corrected" diffusion potentials. The *Fundulus* chorion is too difficult a material for such measurements to be the basis for extensive theoretical discussion. The results are of interest chiefly for their suggestiveness in connection with concrete problems like that of salt antagonism for acid penetration into *Fundulus* eggs.

SUMMARY

By additional measurements of concentration potential differences across the chorion of single *Fundulus* eggs, certain suggestions made in an earlier paper have been confirmed and amplified. In acid-free solutions of KCl, $CaCl_2$, and K_2SO_4 the chorion was found more freely permeable for positive ions than for negative. Its preferential permeability for cations was reduced in all three salts by increasing acidity. In the first two, it was abolished and then reversed, the pH at which equal permeability for anions and cations was reached being about 3.5 in KCl, and 3.9 in $CaCl_2$. In K_2SO_4, however, reversal did not occur down to pH 2.0.

The results suggest an explanation for the mechanism of salt antagonisms for acid penetration into *Fundulus* eggs, a somewhat practical problem. And, from a theoretical standpoint, they seem to furnish evidence that membrane potentials are fundamentally related to diffusion potentials.

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DEVELOPMENT OF THE PARTS OF SEA URCHIN EGGS SEPARATED BY CENTRIFUGAL FORCE

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With rapid centrifuging in the proper medium, the eggs of *Sphærechinus granularis, Parechinus (Echinus) microtuberculatus, Paracentrotus (Strongylocentrotus) lividus*, and *Arbacia pustulosa*, the four commonly occurring sea urchins at Naples, and *Tripneustes (Hipponoë) esculenta* from Bermuda can be broken apart into halves and some of these halves into quarters. The results confirm and complement those previously obtained with the eggs of the Woods Hole sea urchin, *Arbacia punctulata*, which have recently been published (1932). The speed ordinarily used was about 9,000 revolutions per minute (7 cm. radius), and the time necessary to break the eggs apart was from three minutes for *Arbacia pustulosa* to thirty minutes for *Paracentrotus lividus*. The medium used was about half sea water and half 1.1 molal sucrose solution (376 grams sucrose in 1 liter of distilled water) which is isotonic with the eggs; the Δ of the Naples sea water is — 2.2 to — 2.3 whereas that of the Woods Hole sea water is — 1.81; this is also of practically the same density as the eggs themselves, so that they remain suspended in the solution during centrifuging; in the case of the heavier eggs of *Arbacia pustulosa*, a larger proportion of sugar solution was used (3 parts 1.1 molal sucrose to 1 part sea water).

SPHÆRECHINUS GRANULARIS

Normal Egg

The egg of *Sphærechinus granularis* is colorless or slightly yellowish and very granular. It measures 94–102 μ in diameter, averaging 98 μ; the eggs of any one batch are fairly constant in size but different batches vary. The nucleus measures 13 μ in diameter, increasing to 18 μ before it breaks down prior to cleavage. The eggs are usually quite aspherical when laid and remain so for several hours, but become spherical almost immediately on fertilization. The fertilization membrane is well separated from the egg surface, leaving a perivitelline space of 18–20 μ; the ectoplasmic layer is fairly thick, measuring 3–4 μ. The egg develops comparatively slowly, taking about $1\frac{3}{4}$ hours for first cleavage at 16° (50 per cent cleaved). It passes through a typical monaster stage (25–45 minutes after fertilization) and streak stage (50–90 minutes). The density of the egg with jelly is about 1.083 (eggs evenly distributed in 1.1 m sucrose 3 parts: sea water 2 parts) and without jelly about 1.081 (eggs float in 1.1 m sucrose 3 parts: sea water 2 parts, sink in 1.1 m sucrose 5 parts: sea water 4 parts).[2]

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[1] I wish to express my sincere thanks to Dr. Reinhard Dohrn, Director of the Stazione Zoologica, for his interest and courtesy during my stay in Naples, and to the Committee of the American Woman's Table for the use of their research room.

Centrifuged Egg

The unfertilized eggs break apart fairly easily with centrifugal force, usually in 5 minutes with about 9,000 r.p.m. (7 cm. radius) though some batches require longer and some less. The eggs become elongate, then dumb-bell-shaped (Fig. 1 and Photograph 1), then break into two parts with often a connecting strand between (Fig. 2, Photograph 7). If after centrifuging, the strand is very narrow, the parts remain separate; otherwise the strand gradually becomes thicker and the two parts merge into a single sphere.

The stratification is (1) oil, (2) clear layer in which lies the nucleus (3) whitish, loosely-packed granules merging into (4) yellow yolk granules and (5) usually a small clear zone (Fig. 1, Photograph 1). The granules measure approximately in diameter:

Oil	1 μ
White granules	1.5 μ
Yellow yolk granules	1–1.5 μ

Description of Plates

The drawings have been made from living eggs and are magnified about 400 ×. The small circles represent oil drops, the large solid dots (in *Arbacia pustulosa*) red pigment granules, and the stippling represents yolk and other granules, the fine stippling fine granules, and the coarse stippling coarser granules.

Plate I. *Sphærechinus granularis*

1. Stratified whole egg.
2. Whole egg broken into two with a connecting strand between the two parts.
3. White half egg.
4. Granular half egg.
5. White half egg pulling apart.
6. Clear quarter egg.
7. White granular quarter.
8. Granular half egg pulling apart.
9. Upper yolk quarter egg.
10. Lower yolk quarter egg.

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[2] The computations for density of the eggs are based on Wendicke's (1916) figures for the salinity of Naples sea water (37.5 parts salt per 1000 cc.) giving a density of 1.0278 at 16°; and the density of 1.1 molal sucrose, found to be about 1.119 at 16°.

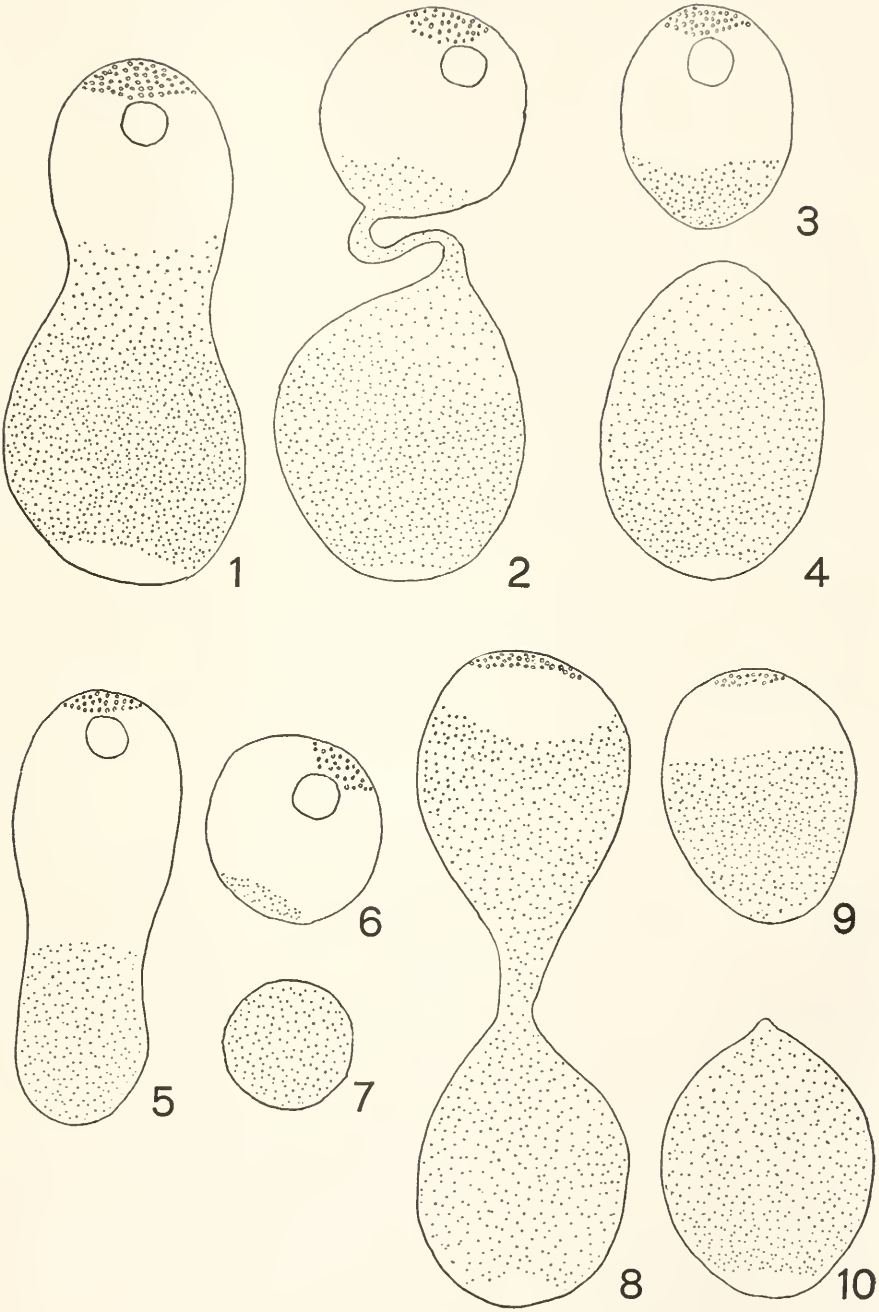
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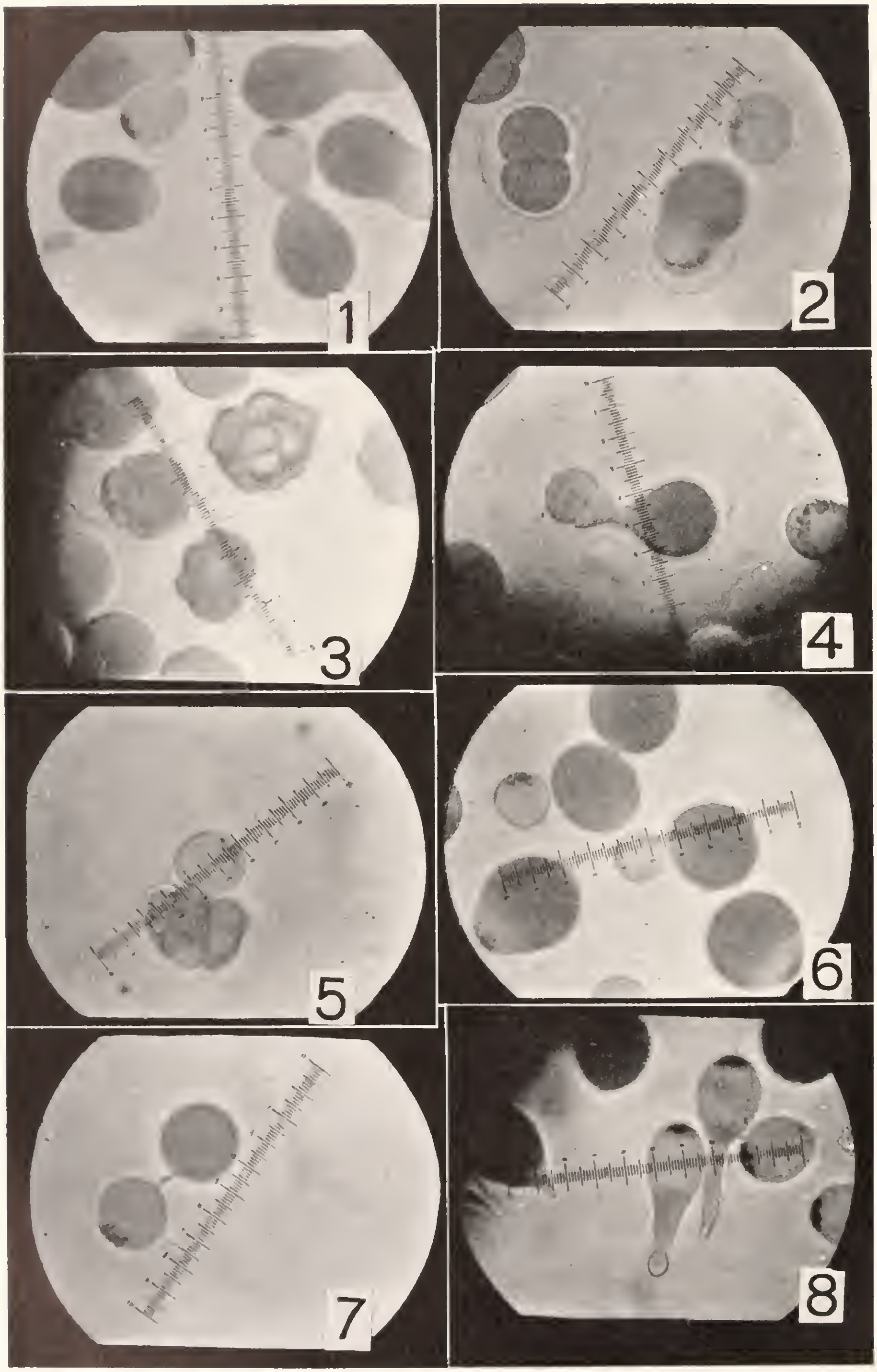
Plate I



Photographs 1–8

The white granules stain purple with methyl green like those in the fifth layer of *Arbacia punctulata* (mitochondria?).[3]

Half and Quarter Eggs

The eggs break apart at 9,000 r.p.m. across the whitish granules, giving two parts, one smaller nucleated half egg containing oil, clear layer and whitish granules (mitochondria?) (Fig. 3 and Photograph 1) and the other half egg, larger, non-nucleate, containing a few whitish granules (mitochondria?) but mostly yellow yolk granules with usually a clear layer at the heavier pole; there is usually also a clear layer at the lighter pole from which granules have been thrown down (Fig. 4 and Photograph 1). In the centrifuge tube, there are not three well-separated layers as in *Arbacia punctulata*, but the layers are contiguous, a whitish layer lying just above a yellowish layer which consists both of dumb-bell-shaped whole eggs above and granular half eggs below. When the eggs are first broken apart, the half eggs are usually not spherical but somewhat elongate, especially the granular half (Photograph 1). In some batches of eggs, without further centrifuging, some of both the clear halves and the yolk halves again become dumb-bells (Figs. 5, 8) and many break apart into quarter eggs. The white half egg separates into an almost clear quarter egg with oil cap and nucleus and a few whitish granules (Fig. 6), and a smaller quarter egg containing only whitish granules (Fig. 7). The granular half egg divides into two somewhat similar parts but the upper quarter egg has a few oil drops at the lighter pole, a clear layer, a few whitish granules and yolk granules (Fig. 9), and the heavier quarter egg has yolk granules and a small clear zone at the blunt end (Fig. 10); the position of the breaking point is often marked by a slight pointed projection. These

Photographs 1–8. *Sphærechinus granularis*

1. Whole egg at right; two half eggs at left.
2. Two-cell stage; granular half at left, white half above, whole egg below.
3. Late segmentation stage; granular half eggs at left; whole egg above.
4. Development of white portion, no development of granular portion of partially separated whole egg.
5. Development of granular portion, no development of white portion of partially separated whole egg.
6. High speed (about 13,000 r.p.m.); granular half much larger than white half. Both half eggs spherical.
7. Low speed (about 5,000 r.p.m.); two half eggs nearly same size; connecting strand between two half eggs.
8. Low speed, white half eggs with tails.

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[3] These granules and similar granules in the other eggs which stain with methyl green, stain also with Janus green B, very kindly given me by E. V. Cowdry. They are without doubt mitochondria (Naples, February 1933).

TABLE I

Size of parts. Diameters in μ; volumes in μ^3 to be multiplied by 10^3.

	Spaerechinus granularis	*Parechinus microtuberculatus*		*Paracentrotus lividus*	*Arbacia pustulosa*	*Tripneustes esculenta*
Speed (7 cm. radius)	9,000 r.p.m.	9,000 r.p.m.	13,000 r.p.m.	9,000 r.p.m.	8,000 r.p.m.	8,000 r.p.m.
Whole Egg						
Diameter	96	102	102	90	77	83
Volume	463.2	555.6	555.6	381.7	239	299.4
Nucleate Half						
Diameter	70	90	96	67	67	77
Volume	179.6	381.7	463.2	157.4	157.4	239
Non-nucleate Half						
Diameter	83	72	58	74	53	47
Volume	299.4	195.3	102.1	212.2	78	54.4
Nucleate Quarter						
Diameter	63				61	
Volume	131.9				118.8	
Non-nucleate Quarter (from nucleate half)						
Diameter	47				38	
Volume	54.4				28.7	
Upper Yolk Quarter						
Diameter	66		48		42	
Volume	150.5		57.9		40.3	
Lower Yolk Quarter						
Diameter	66		45		42	
Volume	150.5		47.7		40.3	

two yolk quarters are of approximately the same size. The smallest quarter egg is the white non-nucleate granular quarter from the white half, and this measures about one-ninth (in one batch one-thirteenth) the volume of the whole egg.

The measurements of the halves and quarters broken apart at 9,000 r.p.m. are given in Table I. There is a slight variation in the relative size of parts in different batches of egg. The data given are for one typical batch.

Relation of Speed to Size of Half Eggs

The relative size of the two half eggs is fairly constant for a constant centrifugal speed. The size of the parts, however, is different for different speeds. With high speed, about 13,000 r.p.m. (7 cm. radius 50 seconds), the granular sphere is much larger than the white sphere (Photograph 6); with low speed, about 5,000 r.p.m. (7 cm. radius 1 hour) the granular half is relatively smaller so that the two halves are almost equal in size (Photograph 7). There is also a difference in the way they break at different speeds. With high speed, the two parts break off as spheres, whereas with low speed there is an elongation of the parts, and often a thick strand of tissue between the two which is left as a tail on one or both parts when separated (Photograph 8). The sizes of parts for one batch of eggs at different speeds are given in Table II.

Development

All of the half and quarter eggs, as well as the elongate and dumb-bell-shaped whole eggs can be fertilized. They throw off fertilization membranes, well separated from the surface as in the normal egg. The membrane follows the contour of the surface even along the strand connecting the two half eggs or along the stalk that is sometimes left after breaking apart (Photograph 8).

In the nucleate half eggs, the stages following fertilization are as in the normal eggs, except for absence of astral rays where granules are absent; and practically as described for the white half eggs of *Arbacia punctulata* (E. B. Harvey, 1932). The first cleavage of a white half egg is shown in Photograph 2; other cleavages follow quite regularly. The blastulæ resulting from the white half egg are at first vacuolated and many remain thus, but many invaginate a day or two later than the normal eggs, form a rudimentary skeleton and pigment spots, but remain almost spherical even for ten days without developing arms. I have never raised any perfectly normal plutei from these half eggs, and they are not very viable after the blastula stage.

Table II

Speed and size of parts. Diameters in μ; volumes in μ^3 to be multiplied by 10^3.

Speed (7 cm. radius)			5,000 r.p.m.		8,000 r.p.m.		13,000 r.p.m.	
	Diam.	Vol.	Diam.	Vol	Diam.	Vol.	Diam.	Vol.
Sphaerechinus granularis egg	102	555.6						
Nucleate half			80	268.0	71	187.4	58	102.1
Non-nucleate half			83	299.4	88	356.8	96	463.2
Parechinus microtuberculatus egg	99	508.0						
Nucleate half			86	332.9	90	381.7	91	394.6
Non-nucleate half			70	179.6	65	143.8	61	118.8
Arbacia pustulosa egg	81	278.3						
Nucleate half			64	137.2	59	107.5	54	82.4
Non-nucleate half			65	143.8	69	172.0	72	195.4
			7,000 r.p.m.					
Paracentrotus lividus egg	90	381.7						
Nucleate half			69	172.0	67	157.4	58	102.1
Non-nucleate half			72	195.3	74	212.2	80	268.0

When the enucleate (merogonic or androgenetic) half eggs are fertilized, the sperm aster develops in the granules in about 10 minutes, a nucleus forms and increases in size as the aster increases, giving a typical monaster stage. This is followed by a " streak " stage, the streak probably being due to the division of the sperm centrosome. The streak fades out, the nucleus enlarges and disappears, and an amphiaster appears, followed by cell division soon after the controls. In aspherical half eggs, the amphiaster lies near the less granular zone, and the division plane divides the cell unequally across the short axis. In spherical eggs, the first division is along the equator (Photograph 2) and this is followed by regular divisions; Photograph 3 shows a late cleavage stage of two of these half eggs and a normal egg. At the fourth division colorless micromeres can sometimes be distinguished. Perfectly normal blastulæ result and become free-swimming about an hour after the controls. These invaginate and acquire a skeleton and pigment about a day later than the controls. Many of the larvæ remain with a well-developed gut, rudimentary skeleton, and pigment spots and do not obtain the typical arms of the pluteus. Some of the larvæ, however, form a complete skeleton and acquire arms and become exactly like the normal plutei except that they are smaller. Some of these small plutei have been kept for 20 days. The androgenetic larvæ are more normal and viable than the larvæ from the nucleated half eggs.

The yellow granular quarter eggs from the granular half eggs develop in the same way as described for the granular half eggs and form regular swimming blastulæ.

The white granular quarter eggs from the white half egg also cleave quite regularly and form swimming blastulæ; some plutei were obtained from these with gut, rudimentary skeleton, and pigment spots. This quarter egg is only one-ninth the volume of the original egg and contains no nucleus, oil or yolk granules and yet can give rise to a pluteus, which though not quite normal has all the fundamental parts of the normal pluteus. The clear quarter eggs may divide equally but long after the controls, and do not develop far. In some of these quarters, the nucleus becomes very large, half the diameter of the cell.

When the elongate or dumb-bell-shaped whole eggs are fertilized, the sperm may enter at any point and an entrance cone is formed. The fertilization membrane starts at this point and follows the contour of the egg. Within a few minutes after the membrane is formed, the egg often pulls away from one or both ends of the membrane, becoming less aspherical (Fig. 11). This probably indicates a decrease in viscosity immediately following fertilization. If the constriction of the dumb-bell is wide, the two pronuclei approach and fuse, usually near the clear end, and the egg divides unequally across the short axis, giving a smaller clear cell and a larger granular cell (Photograph 2), as described for *Arbacia punctulata*. The second division usually comes in at right angles, but sometimes parallel with the first, resulting in a single row of four cells (Fig. 12). By subsequent divisions, a slipper-shaped blastula is formed as in *Arbacia punctulata* (cf. Photograph 17). This gives rise to a normal pluteus.

When the dumb-bell-shaped egg has been broken by the centrifugal force into two half eggs with a thin strand of protoplasm between (Photograph 7), the fertilization membrane forms also over this and usually over both spheres; there is often an appreciable lag (of about 1 minute) in the formation of the membrane over the second sphere. Occasionally the sphere not entered by the sperm does not form a fertilization membrane and does not develop. One or both spheres may receive a sperm. If the sperm enters the nucleate sphere alone, it develops and the granular sphere does not, at least in the cases that I have observed (Photograph 4). If the sperm enters the granular sphere alone, this sphere may develop without the other (Photograph 5) or both spheres may develop (Figs. 13, 14). If both spheres receive a sperm, both usually develop. The two parts develop quite independently, even the first cleavage not being synchronous (Fig. 14). The protoplasmic strand connecting the two half eggs is sometimes broken early in development (Fig. 13). Both parts often develop into blastulæ forming twins swimming around together.

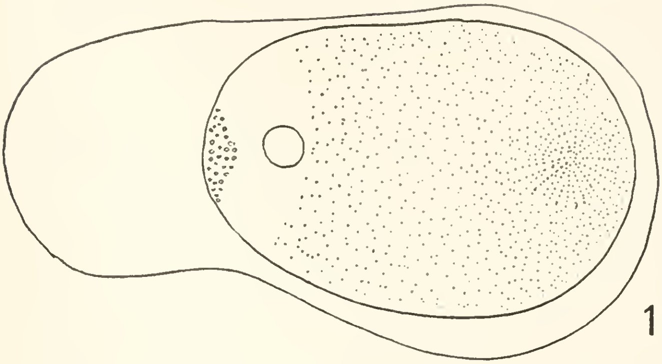
PARECHINUS (ECHINUS) MICROTUBERCULATUS

Normal Egg

The egg of *Parechinus* (*Echinus*) *microtuberculatus* is colorless, not very granular, but quite clear and transparent. Its diameter measures 96–113 μ, averaging 102 μ; the nucleus measures 13 μ. The eggs are quite aspherical when laid and remain so for several hours, but round up immediately on fertilization. The fertilization membrane is well separated from the egg surface, leaving a perivitelline space of 21 μ; the ectoplasmic layer when fully formed measures 2–3 μ. First cleavage takes place in 70 minutes at 16°. The density of the egg with jelly is about 1.074 (sometimes float, sometimes sink in 1.1 m sucrose 1 part: sea water 1 part), and without jelly about 1.072 (float in 1.1 m sucrose 1 part: sea water 1 part).[2]

Plate II. *Sphærechinus granularis*

11. Egg slipped back from fertilization membrane, soon after fertilization.
12. Four-cell stage, second division plane parallel with first.
13. Independent development of two halves of egg partially separated, connecting strand broken.
14. Another egg, later stage, connecting strand still present.



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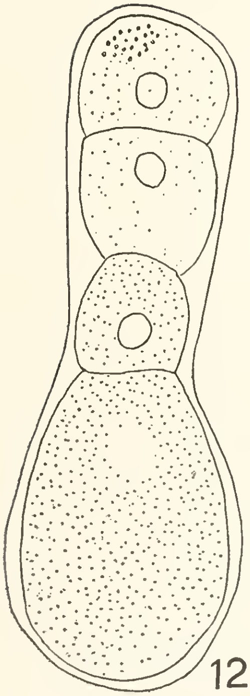

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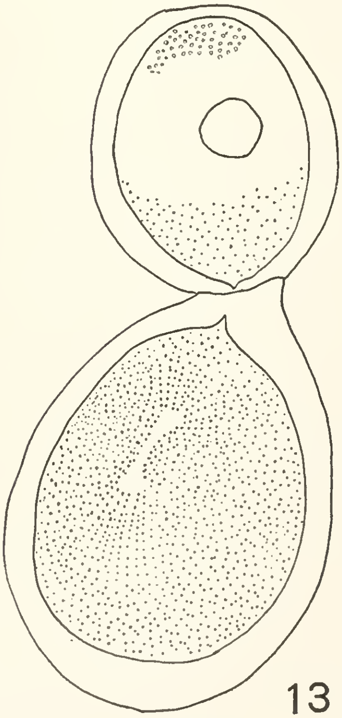

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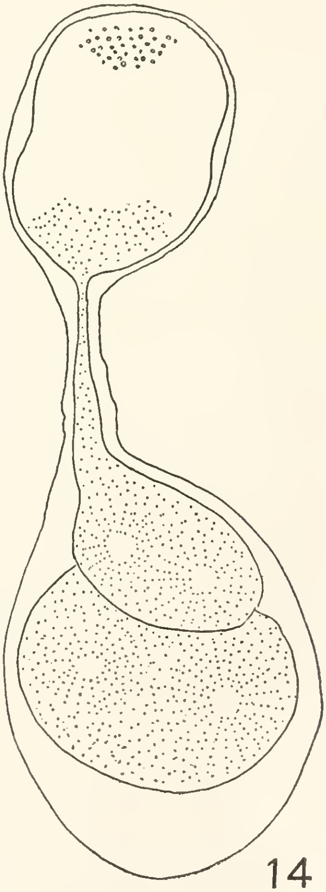

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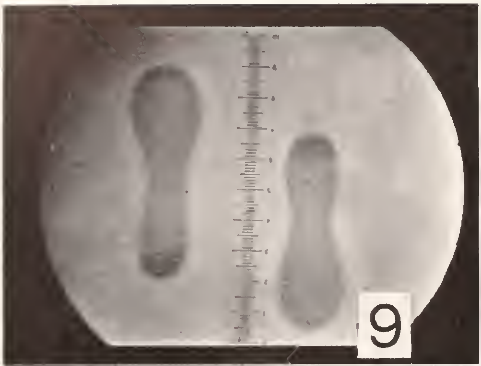
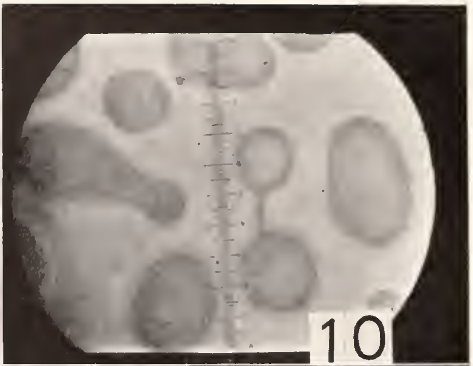
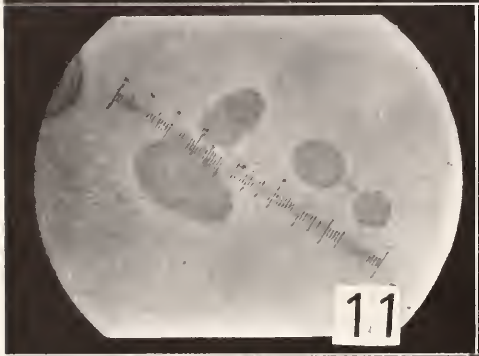
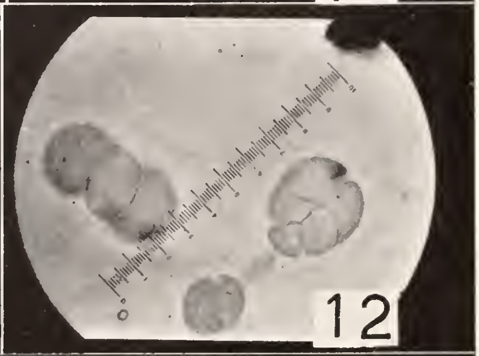
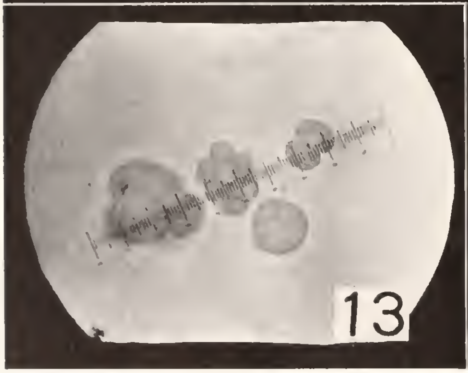
Plate II

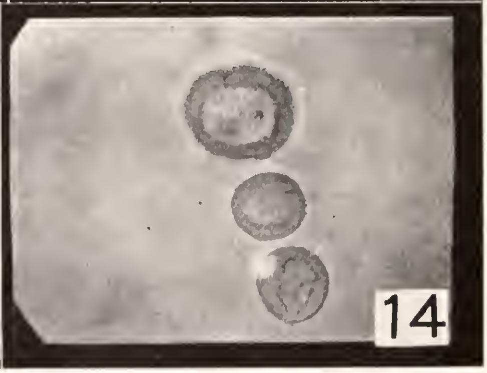












Photographs 9-14. *Parechinus* (*Echinus*) *microtuberculatus*

9. Whole egg pulling apart; cap of fine yellow granules at one end.

10. Egg pulled apart with connecting strand.

11. Two half eggs at left; yellow granular half pulled apart into quarter eggs with strand between, at right.

12. Four-cell stage of whole egg at left, white half at right, yellow granular half below, with connecting strand between the two halves.

13. Cleavage of white half at left, granular half in center; two quarter eggs (from yellow granular half) at right connected by a strand.

14. Blastula of whole egg above; of two yellow granular half eggs below.

Centrifuged Egg

When the egg is centrifuged at about 9,000 r.p.m. the stratification is (1) oil (2) coarse white granules at the lighter end in which lies the nucleus (3) almost clear zone (4) very small, closely-packed yellowish granules (Fig. 15, Photograph 9). This egg (and that of *Tripneustes*) differs from the others studied in having granules lighter than the clear zone, which lie under the oil cap, and the nucleus lying among these granules instead of in a clear zone. A similar condition has been described for *Tubifex rivulorum* by Parseval (1922). The relative area of coarse white granules and clear zone differs in different lots owing probably to the amount of packing of the granules. The granules measure approximately:

Oil	0.7 μ
Coarse white granules	2 μ
Fine yellow granules	0.4 μ

The small yellow granules at the heavy end stain purple with methyl green (mitochondria?).

Half and Quarter Eggs

The eggs break apart at about 9,000 r.p.m. usually in 8 minutes with often a connecting strand between the two parts (Photograph 10). The larger sphere contains oil, nucleus, and coarse white granules, and the smaller sphere consists of an almost clear half and a yellowish half composed of the fine yellow granules (mitochondria?). The measurements for a typical lot at 9,000 r.p.m. are given in Table I.

In some batches of eggs the small enucleate halves formed dumbbells and broke into two spheres, some connected with each other by a thin strand of tissue (Photograph 11). The halves and quarters obtained in one batch broken at about 13,000 r.p.m. are given in Table I.

Speed and Size

The size of the two half eggs varies somewhat with the centrifugal speed. In all the other species, the higher speed increases the size of the granular or heavier sphere. In *Parechinus* the heavier sphere decreases in size with high speed, and increases with low speed. In Table II are given the data for one lot of eggs.

Development

All of the half and quarter eggs can be fertilized and throw off well-separated membranes like the whole egg. They all cleave and form swimming blastulæ (Photographs 12, 13, 14); and some dwarf plutei were raised from the enucleate halves. The elongate whole egg when fertilized often slips away from one or both ends of the membrane, becoming less aspherical as noted for *Sphærechinus.* When the two parts are connected by a strand of tissue, they often develop independently (Photograph 12) and form twin blastulæ. One part may, however, develop and the other part remain undeveloped, just as described for *Sphærechinus.*

Paracentrotus (Strongylocentrotus) lividus

Normal Egg

The egg of *Paracentrotus (Strongylocentrotus) lividus* is clear and transparent and not very granular. It is colorless except for an orange or reddish band that encircles the egg just below the equator; this is very inconspicuous in some batches of eggs and quite noticeable in others as noted by many other observers. The diameter of the egg is 83–95 μ, averaging 90 μ; the nucleus measures 12 μ, increasing to 16 μ before cleavage. The eggs are not very spherical when laid, but are more so than are those of *Sphærechinus* and *Parechinus.* The fertilization membrane is fairly well separated from the egg, leaving a perivitelline space of 10–12 μ. The ectoplasmic layer is thin, 1–2 μ. First cleavage takes place in 1½ hours at 16°. The density of the egg with jelly is about 1.083 (eggs evenly distributed in 1.1 sucrose 3 parts: sea water 2 parts); without jelly about 1.079 (evenly distributed in 1.1 m sucrose 5 parts: sea water 4 parts).[2]

Centrifuged Egg

This egg is much more difficult to break apart than either *Sphærechinus* or *Parechinus;* it takes about 30 minutes at 9,000 r.p.m. and none of the eggs were broken into quarters. The stratification is (1) oil (2) clear layer in which lies the nucleus (3) coarse granules (4) fine granules (Fig. 16, Photograph 15). The granules measure approximately:

Oil	0.8 μ
Coarse granules	1.5 μ
Fine granules	0.5–1.5 μ

The fine granules stain purple with methyl green (mitochondria?). The orange band is not thrown down in the mature eggs by centrifugal

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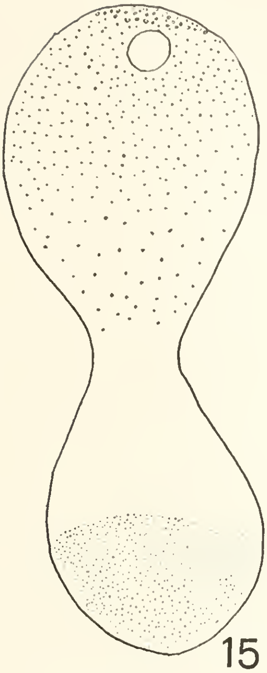
Plate III

15. *Parechinus (Echinus) microtuberculatus,* stratified whole egg.
16. *Paracentrotus (Strongylocentrotus) lividus,* stratified whole egg.
17. *Paracentrotus,* tripartite egg, all three parts with sperm asters.
18. Same developed into triple blastula.
19. *Arbacia pustulosa,* stratified whole egg.
20. *Tripneustes (Hipponoë) esculenta,* stratified whole egg.



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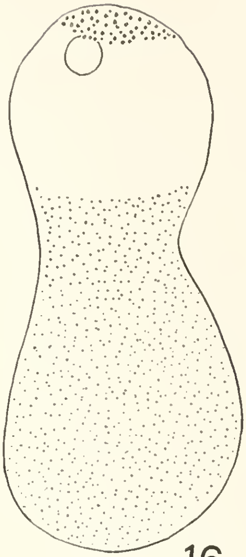

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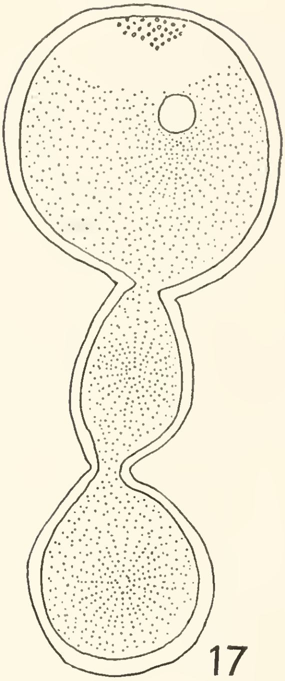

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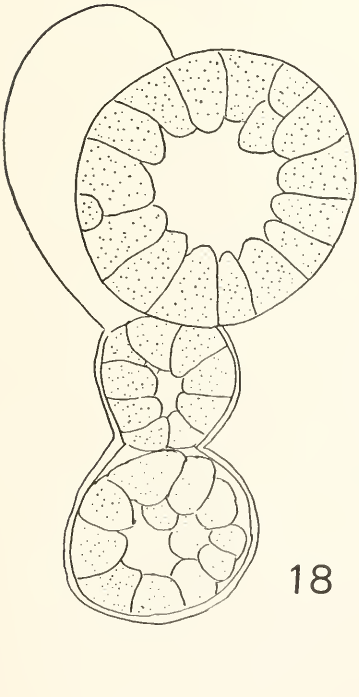

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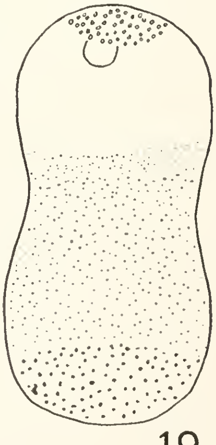

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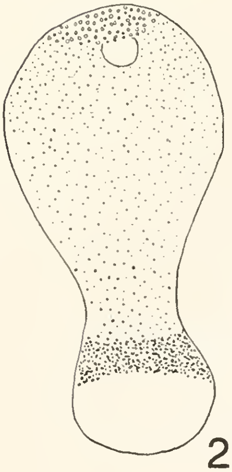

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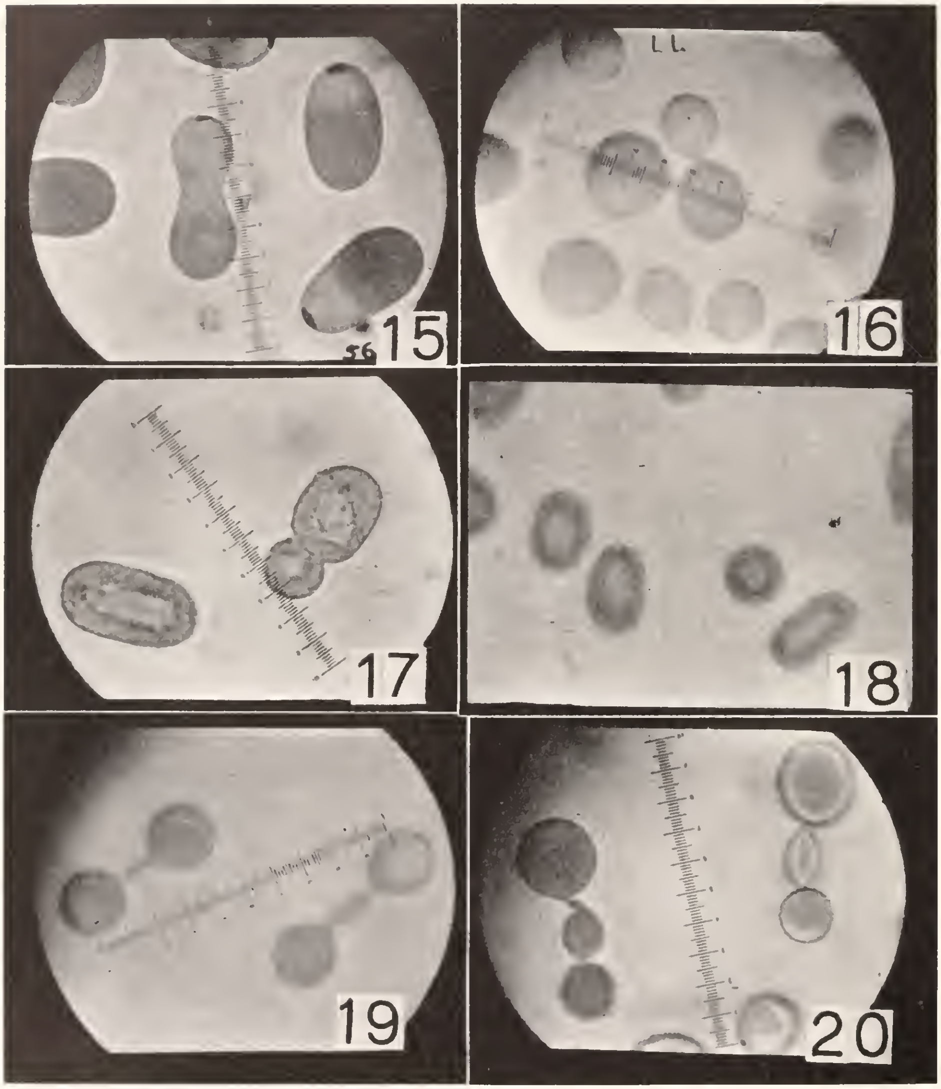
Plate III



Photographs 15–20. *Paracentrotus* (*Strongylocentrotus*) *lividus*

15. Whole egg.
16. Two half eggs in center; high speed.
17. Blastulæ of whole eggs, one at right from incompletely separated egg.
18. Blastula of granular half egg above at right, whole egg below it.
19. Two half eggs connected by strand; low speed.
20. Tripartite eggs. Egg has been separated into three parts instead of two.

force, and there is no massing of pigment at the heavy pole such as occurs in *Arbacia*. The band remains in place and is merely stretched as the egg elongates. It may be, therefore, in any relation to the stratification: parallel, diagonal, or perpendicular to it. In immature eggs with germinal vesicle, the red pigment goes to the light pole either with the oil or a little below it.

Half Eggs

The eggs break apart at about 9,000 r.p.m. into a smaller nucleated sphere consisting of oil, clear layer, and coarse granules, and a larger non-nucleate sphere consisting of a few coarse granules and fine granules (mitochondria?) (Photograph 16). The two spheres are often connected by a narrow strand (Photograph 19).

Speed and Size

The size of the two spheres varies with the centrifugal speed. The higher speed increases the size of the granular sphere as in *Sphærechinus* (Photograph 16). With slow speed the granular sphere is smaller and more nearly equal in size to the clear sphere (Photograph 19). The sizes with different speeds are given in Table II.

Development

Both the half eggs can be fertilized, throw off fertilization membranes well separated from the surface, and form swimming blastulæ (Photograph 18) and gastrulæ with skeleton and pigment. When the halves are connected by a strand, one or both parts may develop. The elongate whole egg tends to slip away from the fertilization membrane just after fertilization as in *Sphærechinus* and *Parechinus*. It develops into a slipper-shaped blastula, or, if constricted by centrifuging, into a more or less double blastula (Photographs 17, 18). Invagination always takes place in the reddish zone no matter where located. The gastrulæ are therefore of various shapes, elongate in the axis of the gut or flattened in this axis. There seems no doubt that the axis of the embryo depends upon the location of the red pigment band and not upon the stratification of materials or the elongation of the egg produced by centrifugal force. Owing to the difficulty in seeing the red pigment band in the half eggs, it was not possible to determine whether some of these entirely lacked the pigment band and therefore failed to gastrulate.

Triple Eggs

In one set of eggs centrifuged at 9,000 r.p.m. the egg constricted not into two spheres but into three of very constant and definite size: a large sphere, a medium-sized sphere, and a small sphere between the two (Photograph 20). When fertilized, the membrane formed around all three parts and in each a sperm aster developed, indicating that each part was fertilized by a separate sperm (Fig. 17). Some of these developed into triplet blastulæ, the three parts developing independently and swimming together (Fig. 18). In one case, the two larger parts formed gastrulæ.

Arbacia pustulosa

Normal Egg

The egg of *Arbacia pustulosa* is very heavily pigmented, even more reddish than that of *Arbacia punctulata*. The diameter of the egg is 77–81 μ, averaging 79 μ; the nucleus measures 10 μ. The fertilization membrane is closely adherent to the egg, the perivitelline space measuring only 1–2 μ. The ectoplasmic layer is quite thick (3 μ on fertilization) and it is possible to tell by measuring the same egg before and after fertilization that it is formed on fertilization outside of and in addition to the red sphere. First cleavage takes place in 1 hour 50 minutes at 16°. The egg is quite dense; with jelly its density is about 1.101 (eggs evenly distributed in 1.1 m sucrose 4 parts : sea water 1 part), without jelly about 1.096 (eggs evenly distributed in 1.1 m sucrose 3 parts : sea water 1 part).[2]

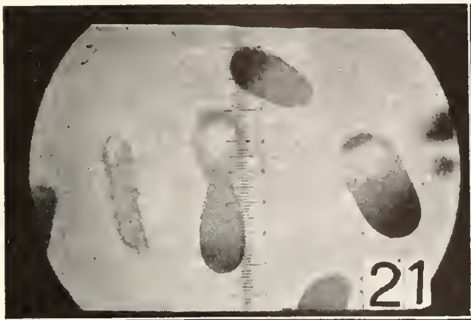
Centrifuged Egg

This egg breaks apart very readily and forms three layers in the centrifuge tube, distinct and well separated—the white nucleated spheres on top, the red non-nucleate spheres at the bottom, and dumb-bell-shaped whole eggs in the middle. The stratification is just as it is in *Arbacia punctulata* (1) oil, (2) clear layer in which lies the nucleus (3) fine granules or fifth layer (4) yolk granules and (5) red pigment (Fig. 19, Photograph 21). The granules measure approximately:

Oil	0.8 μ
Granules of fifth layer	0.5 μ
Yolk granules (polyhedral)	0.6–1 μ
Pigment	1.5 μ

Photographs 21-28. *Arbacia pustulosa*

21. Whole egg in center. Pigmented half above.
22. Two half eggs.
23. Pigmented half egg and the two quarters into which it breaks.
24. Low speed. White half larger than pigmented half.
25. High speed. Pigmented half larger than white.
26. Two-cell stage of pigmented half.
27. Blastula of white half above, of dumb-bell-shaped whole egg at right, and also below.
28. *Tripneustes (Hipponoë) esculenta.* Two half eggs within jelly.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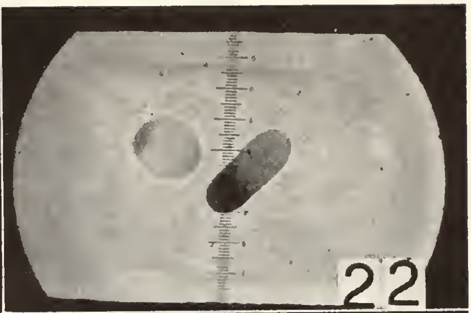
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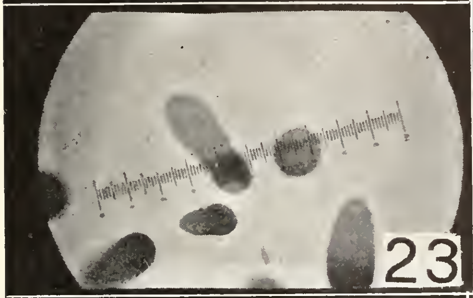
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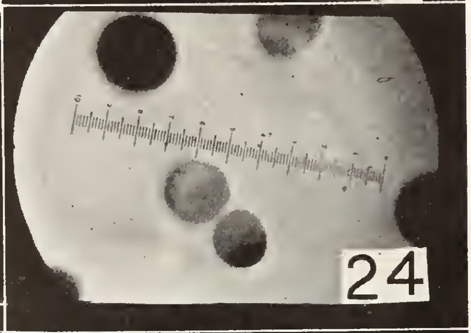
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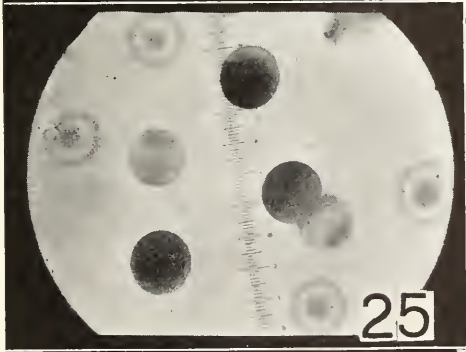
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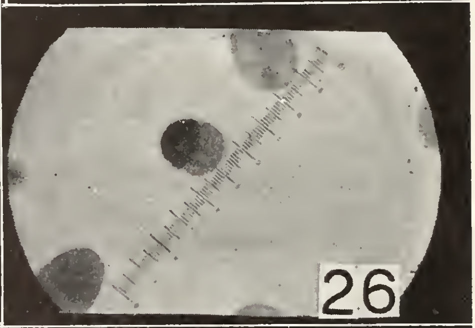
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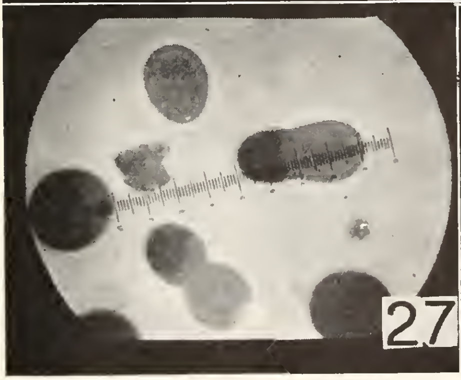
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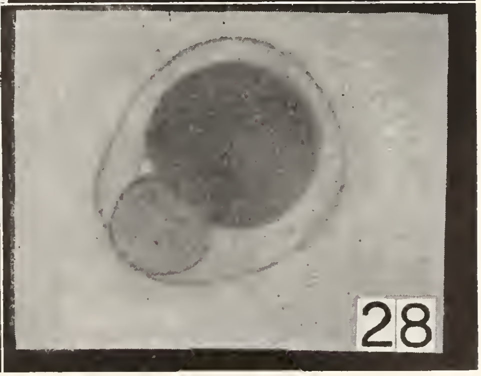
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Photographs 21–28

The granules of the fifth layer stain purple with methyl green (mitochondria?).

Half and Quarter Eggs

The eggs are usually broken in 3 minutes at 9,000 r.p.m. across the yolk into a clear sphere containing oil, nucleus, clear layer and fifth layer (mitochondria?) and a little yolk; and a yolk portion containing yolk and pigment (Photographs 21–25). In some batches of eggs, the white sphere is a little larger than the red when centrifuged at 9,000 r.p.m. and in other batches the red sphere is a little larger than the white. The diameters of two typical lots centrifuged at 9,000 r.p.m. were: (1) whole egg 77 μ, nucleate half 62 μ, non-nucleate 60 μ; and (2) whole egg 78 μ, nucleate half 61 μ, non-nucleate 64 μ.

In one batch of eggs, broken apart at about 8,000 r.p.m., both the half eggs broke into quarters (Photograph 23, yolk quarters). The sizes of all halves and quarters are given in Table I.

Speed and Size

When the eggs are centrifuged at low speed, the white sphere is somewhat larger than the red sphere (Photograph 24); as the speed increases, the size of the red sphere increases, until at very high speed (about 13,000 r.p.m.) it is considerably larger than the white sphere (Photograph 25). At high speeds the egg breaks apart, giving two spherical half eggs (Photograph 25); the materials are not entirely segregated and the fifth layer is not very well formed. At low speeds, a much more definite segregation of materials takes place, the fifth layer is well formed, the two parts, especially the yolk half, become elongate before breaking apart (Photograph 21), and a tail is often left on the white sphere. The sizes of the two half eggs at different speeds in one lot of eggs are given in Table II.

Development

The deformed whole eggs and the half eggs of *Arbacia pustulosa* develop exactly as described for *Arbacia punctulata* (E. B. Harvey, 1932). The white nucleate halves divide quite regularly and give quite normal plutei with skeleton and arms but dwarf and lacking pigment. The red enucleate halves often develop without division planes coming in or represented by notches, but sometimes normal cleavage takes place (Photograph 26); and blastulæ and a few plutei with skeletons have been raised. These are not so normal or viable as the white halves. The whole eggs divide as in *Arbacia punctulata*, giving slipper-shaped blastulæ (Photograph 27) and later normal plutei.

Tripneustes (Hipponoë) esculenta

Normal Egg

The egg of *Tripneustes (Hipponoë) esculenta*, obtained in Bermuda in November, is slightly yellowish and very granular, similar in appearance to that of *Sphærechinus granularis*. It measures 82–86 μ, and is quite spherical when laid. The nucleus measures about 11.5 μ. The fertilization membrane adheres so closely to the surface that it is difficult to detect, leaving no perivitelline space. The ectoplasmic layer is very thin. The eggs develop slowly, taking 1½ hours for first cleavage after fertilization at 22° C.

Centrifuged Egg

The unfertilized egg breaks apart in about 15 minutes at about 8,000 r.p.m., 7 cm. radius. The stratification is usually (1) oil (2) coarse granules (3) fine yellow granules (4) clear layer (Fig. 20). The nucleus lies among the coarse granules under the oil cap. The granules measure approximately:

Oil	0.7 μ
Coarse granules	1.2 μ
Fine granules	0.7 μ

The fine yellow granules stain purple with methyl green (mitochondria?).

This egg, together with that of *Parechinus*, differs from the other sea urchin eggs, as well as most other eggs (except *Tubifex*), in having coarse granules under the oil, and the clear layer below. The coarse granules must, therefore, be lighter than the fluid medium of the egg. In *Parechinus*, the mitochondrial (?) granules are heavier than the medium, leaving the clear layer above these; in *Tripneustes* these granules, as well as the yolk granules, are lighter than the medium, leaving the clear layer below. By immersing the eggs in a medium of 70 per cent sea water: 30 per cent fresh water and centrifuging in this medium (with sucrose solution below), a very definite clear layer was formed under the oil. The medium in the egg, having been made less dense by the addition of water, is now lighter than the granules, and the granules sink to the heavier pole. In some batches of normal eggs and in some eggs of other batches, the clear layer is found, as in the eggs treated with dilute sea water, beneath the oil instead of at the heavy pole. When these eggs are centrifuged in concentrated sea water (1 gram NaCl + 100 cc. sea water), there is no clear layer beneath the oil. The medium of the egg having been made more dense, the granules now rise toward the lighter pole.

It may be noted in passing that eggs in dilute sea water break apart much less readily than control eggs, and those in concentrated sea water much more readily.

Half Eggs

The *Tripneustes* egg breaks at about 8,000 r.p.m. (7 cm. radius) into two very unequal parts, usually a large sphere containing oil, nucleus, and coarse granules, and a very small sphere containing fine yellow granules (mitochondria?) and clear layer. The two parts are usually connected by a strand of tissue and are held together by the surrounding jelly, as shown by staining with Janus green (Photograph 28). The sizes of the parts are given in Table I.

Development

Both the nucleate and non-nucleate half eggs can be fertilized, the fertilization membranes closely adhering to the surface. The large sphere divides equally, sometimes through the oil cap, sometimes elsewhere. Swimming blastulæ develop a little later than those from whole eggs, then form gastrulæ which later obtain a skeleton and pigment. The small spheres develop with only the male nucleus, sometimes division planes come in, but usually only the nuclei divide until blastulæ are formed. These swim still later than those from the larger half egg. The two halves usually break their connecting strand when becoming free-swimming. The larger halves are much more viable and normal than the smaller halves.

Cross-fertilization

Some experiments were done with fertilizing the half and quarter eggs of one species with the sperm of another species. In general it was found that when cross fertilization is not possible (in sea water) with normal eggs, it is also not possible with centrifuged eggs nor with half and quarter eggs. When cross-fertilization is possible with normal eggs, it takes place in about the same percentage (sometimes slightly more) in the deformed whole eggs and in the half and quarter eggs, both nucleate and enucleate. The best results were with *Sphærechinus* ♀ and *Paracentrotus* ♂, where many quite normal cleavages occurred in all types of eggs. Some of these hybrids from fertilized enucleate half eggs were raised to plutei with skeletons and have lived as long as seven days.

DISCUSSION

A comparison of the results obtained in five species of sea urchin brings out several facts. On the one hand, there are marked differences in the normal egg of the different species. They differ in size, density, size of nucleus, size of perivitelline space, thickness of ectoplasmic layer, and rate of development. On centrifuging, there is a difference in the distribution of visible materials (except in the two species of *Arbacia*), in the force necessary to break the eggs apart, in the size of the corresponding portions and in their viability after fertilization. On the other hand, there are certain similarities in the eggs. All the eggs stratify when centrifuged, become dumb-bell-shaped, and break into two spheres of very definite size. The granules which are moveable by centrifugal force, though differing in relative size and weight, are probably fundamentally the same in all the eggs: oil, yolk granules, and another kind of granules, probably mitochondria; in the two species of *Arbacia* there are pigment granules in addition.

When the eggs are broken by centrifugal force into halves or quarters, all the parts can be fertilized, and form fertilization membranes characteristic of the particular normal whole egg. All the parts are capable of development, some of the parts of complete development, at least as far as plutei. The female nucleus does not seem to be essential for development, since in some cases the half lacking this nucleus (androgenetic) develops better than the half containing it. I have found no evidence of any localization of organ-forming material around the nucleus, as found by Harnley (1926). The visible granules do not seem to be the essential materials for development, since the fundamental organs such as gut and skeleton can be laid down in the absence of any one special type of granule. These granules seem to be accessory rather than essential materials in development; the essential material for development seems to be the "ground substance." This agrees with the conclusions of many others who have worked on centrifuged eggs (especially Morgan) and with the work of E. B. Wilson (1929) on the centrifuged fragments of *Chætopterus* eggs. There seems to be little localization of visible organ-forming materials in the unfertilized sea urchin egg, a conclusion reached also by Plough (1929) and by Tennent, Taylor, and Whitaker (1929).

The pigment band characteristic of the *Paracentrotus* egg belongs to a different category from the other visible materials, as this is *not* displaced by centrifugal force and *does* indicate the place of invagination, whether or not it is actually gut-forming material. This agrees with the earlier work of Boveri (1901) and the later work of Hörstadius (1928) on this egg, definitely locating the gut-forming material in this band.

Summary

1. The unfertilized eggs of *Sphærechinus granularis, Parechinus* (*Echinus*) *microtuberculatus, Paracentrotus* (*Strongylocentrotus*) *lividus* and *Arbacia pustulosa* from Naples and of *Tripneustes* (*Hipponoë*) *esculenta* from Bermuda have been stratified and broken into halves and some of these halves into quarters by strong centrifugal force.

2. The relative size of the halves and their granular content is fairly constant for a definite centrifugal speed, but the size varies with the speed.

3. The visible granules are stratified with centrifugal force; the stratification is differently arranged in the different species but always includes oil, yolk and mitochondria (?). The pigment band of the *Paracentrotus* egg is not displaced but only stretched.

4. When fertilized, all of the halves and quarters, both nucleate and non-nucleate, form fertilization membranes and develop, some into normal dwarf plutei.

5. When the half eggs are connected by a strand of tissue, either one or both spheres may be fertilized by a sperm and either one or both may develop.

6. In one batch of *Paracentrotus* eggs, the eggs constricted into three parts instead of two, and each part received a sperm and developed.

7. Half and quarter eggs, both nucleate and non-nucleate, can be cross-fertilized in approximately the same percentage as the whole eggs. Some of the cross-fertilized non-nucleate halves were raised to plutei.

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STUDIES ON THE BIOLOGY AND CHEMISTRY OF THE GULF OF MAINE

I. Chemistry of the Waters of the Gulf of Maine in August, 1932

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The Woods Hole Oceanographic Institution [1]

The hydrographic features of the Gulf of Maine—temperature, salinity, and the dominant concentration of its waters—are now known broadly speaking (4) (5). The same may be said of the general occurrence of its principal inhabitants (3). The final goal of such studies, the explanation of the natural economy of these inhabitants in terms of their physical and chemical environment, lies ahead. It is to be sought not so much by the generalized surveys of the area as by more detailed study of the physical and, more especially, of the chemical changes in the water from season to season at selected locations, combined with simultaneous observations on the occurrence, abundance, and distribution of the animals and plants.

Oceanic biologists in various parts of the world have devoted much attention during the past few years to the factors and events that control the biologic fertility of the seas. Parallelism has so repeatedly been established between variations in the abundance of phytoplankton on the one hand and in the available supply of plant-nutrients on the other, that a causative relationship is now universally accepted. Much of detail is yet to be learned not only regarding the nutritive requirements of different groups of planktonic plants, but equally as to the situation existing with regard to the cycle of limiting substances in different parts of the sea.

The Gulf of Maine appears to be a particularly favorable area for such detailed studies. Within a circumscribed region of ready accessibility there occurs a variety of hydrographic conditions, in many ways representative of boreal seas as a whole. These range from the turbulent shallow waters overlying the offshore banks to the stable and stratified waters which develop in the deeper portions of the Gulf during the summer. Within this area exceptionally large ranges of temperature occur, both seasonal and in correlation with the depth of the water. Furthermore, the deeper portions of the Gulf appear to be fed by a tongue of water originating at even greater depths offshore. This water will be shown from its chemical content to be characteristic of the more stagnant waters of the deep sea. Conditions are consequently favorable for the examination of the nutrient cycle in all its phases.

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[1] Contribution No. 8, Woods Hole Oceanographic Institution.

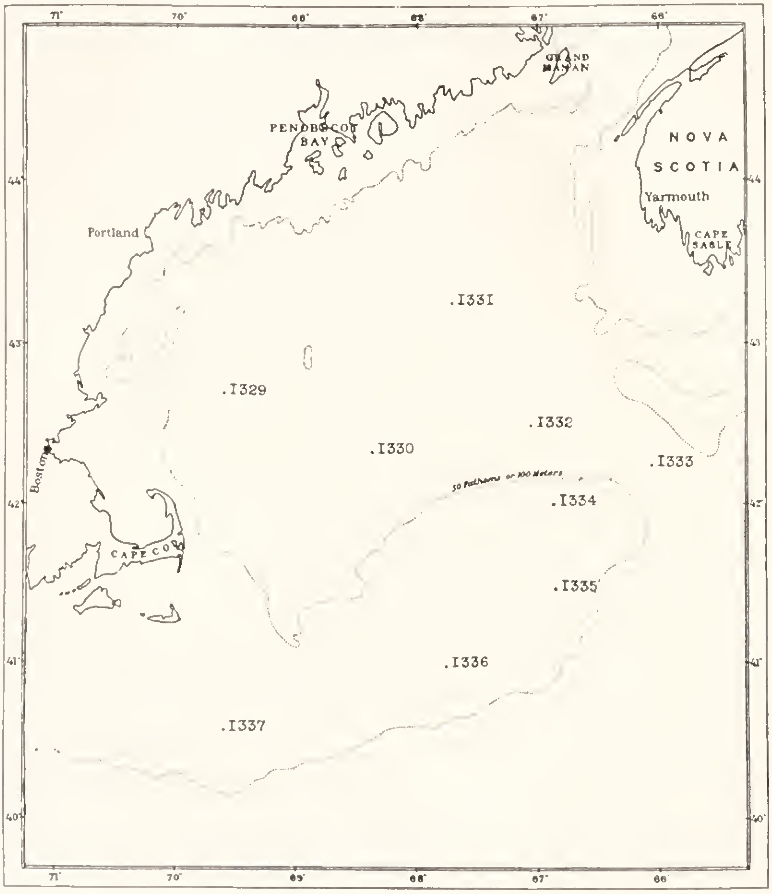
71°
70°
69°
68°
67°
66°
44°
43°
42°
41°
40°
GRAND MANAN
PENOBSCOT BAY
NOVA SCOTIA
Yarmouth
CAPE SABLE
Portland
Boston
CAPE COD
.1331
.1329
.1332
.1330
.1333
30 Fathoms or 100 Meters
.1334
.1335
.1336
.1337

FIG. 1. Locations of "Atlantis" Stations 1329–1337, August 2–5, 1932.

During the month of August, 1932, a cruise of the research ship "Atlantis" afforded an opportunity for the simultaneous study of the quantitative distribution of phytoplankton, of bacteria, and of the most obviously important nutrient substances of the sea water. Stations were made in two regions of striking contrast, the deep basin of the Gulf on the one hand, and the waters over its shoal southern rim (Georges Bank) on the other. The location of the stations is charted in Fig. 1 and their position recorded in Table I.

The present paper deals with the chemical observations made during this cruise. The results of the study of phytoplankton and bacteria are presented in the succeeding communications.

The chemical program of the cruise consisted in the determination of phosphate, nitrate, nitrite, and oxygen in the water at various depths from all stations. The methods used were those which have now become rather well standardized: for phosphate, the colorimetric molybdic acid method successfully used by Atkins and many others (1) (2) (3) (8); for nitrate, the colorimetric method of Harvey (7) (8), using reduced strychnine; for nitrite, the Griess-Ilosvay method, but substituting the diethyl derivative of alpha-naphthylamine; for oxygen, Winkler's iodimetric titration. All analyses were carried out on ship-board, as soon as possible after the water samples had been taken. The oxygen and phosphate analyses were made by Mr. T. S. Greenwood.

TABLE I

Location of Stations

Station No.	Date	Lat. N.	Long. W.
1329	August 2, 1932	42° 42′	69° 36′
1330	August 2, 1932	42° 19′	68° 22′
1331	August 3, 1932	43° 14′	67° 45′
1332	August 3, 1932	42° 28′	67° 03′
1333	August 4, 1932	42° 15′	66° 04′
1334	August 4, 1932	42° 00′	66° 54′
1335	August 4, 1932	41° 26′	66° 53′
1336	August 5, 1932	40° 57′	67° 49′
1337	August 5, 1932	40° 35′	68° 42′

From the chemical standpoint as well as the hydrographic the stations may be divided into two groups; the first five, Nos. 1329–1333, fairly deep, in the Gulf of Maine; and the last four, Nos. 1334–1337, much shallower, on Georges Bank. A more thorough mixing is characteristic of the latter group, a less pronounced thermocline and, as a consequence, a more nearly homogeneous water mass, top to bottom.

The data are given in Table II and plotted in depth-concentration curves, Figs. 2, 3, and 4. In the latter, the scale is different for each of the five items, and is so chosen as to make the curves most nearly correspond with each other. In the tables and curves the lower limit for nitrite nitrogen is taken as approximately 0.4 mg./m.^{3}; that is, the water is considered nitrite-free when amounts less than this are present, although with care it is possible to detect perhaps half this amount.

TABLE II

Hydrographic and Chemical Data for Stations in the Gulf of Maine and on Georges Bank

	Depth	Temp.	Salinity	Nitrate N	Nitrite N	Phosphate PO_4	Oxygen content	Oxygen saturation
	meters	° *C.*		*mg./m.*^{3}	*mg./m.*^{3}	*mg./m.*^{3}	*cc.*	*per cent*
Station 1329 August 2, 1932	1	19.19	32.00	25	0	18	5.40	98
	10	16.81	32.21	24	0	13	6.30	110
	20	11.02	—	35	0	11	7.27	113
	30	8.16	32.45	34	0	49	7.44	108
	40	6.2	32.63	120	1.3	77	6.63	94
	50	5.15	32.92	175	1.0	102	6.39	89
	60	5.2	32.79	195	1.0	100	6.34	88
	75	4.83	32.92	240	1.0	115	6.10	84
	100	4.59	33.19	275	0.8	85	5.79	80
	240	6.30	34.23	425	1.1	96	4.38	63
Station 1330 August 2, 1932	1	19.81	32.70	21	0	20	5.56	102
	10	19.43	32.20	21	0	11	5.57	102
	19	13.11	32.54	25	0	11	6.99	114
	29	8.80	32.66	27	0	36	7.26	109
	39	6.03	32.92	105	1.6	—	6.75	96
	48	5.01	32.92	170	0.4	43	6.51	90
	63	4.65	32.92	190	0.4	45	6.46	89
	77	4.45	33.01	240	0.7	54	6.34	87
	101	4.47	33.12	250	0	59	6.17	85
	189	5.81	34.23	390	0	82	4.76	68
Station 1331 August 3, 1932	1	16.57	32.75	28	1.2	12	6.07	106
	10	13.87	32.39	27	1.3	8	6.63	110
	19	9.65	33.03	35	2.1	9	7.24	111
	29	8.54	32.77	75	2.9	18	6.53	97
	39	6.79	32.92	135	3.6	17	6.07	87
	48	5.81	32.92	150	1.7	18	5.94	83
	58	6.00	33.12	210	1.4	19	5.78	82
	72	5.68	33.13	225	1.2	80	5.67	80
	97	5.53	32.97	285	0.9	—	5.43	76
	194	6.67	34.37	350	1.6	—	4.47	65
Station 1332 August 3, 1932	1	17.67	32.48	22	0	22	5.90	104
	9	17.60	32.72	20	0	22	5.90	104
	19	13.90	32.34	16	0	23	6.66	110
	28	11.09	32.66	27	1.5	36	6.95	107
	38	9.67	32.57	27	1.4	39	6.81	104
	47	8.65	32.59	60	2.7	50	6.82	102
	56	7.31	32.84	155	3.8	67	6.40	93
	71	6.13	33.68	235	1.5	86	5.94	84
	94	6.13	33.89	265	1.2	100	5.39	77
	188	7.17	34.85	450	1.8	132	4.27	63
	310	6.33	34.85	500	1.1	138	4.48	64

TABLE II (*Continued*)

	Depth	Temp.	Salinity	Nitrate N	Nitrite N	Phosphate PO_4	Oxygen content	Oxygen saturation
	meters	° *C.*		*mg./m.*^{3}	*mg./m.*^{3}	*mg./m.*^{3}	*cc.*	*per cent*
Station 1333 August 4, 1932	1	17.31	32.43	16	0	22	5.78	102
	9	16.81	32.34	20	0	28	6.00	104
	17	9.96	32.48	20	0	32	6.92	106
	26	7.57	——	80	3.5	36	6.68	98
	35	6.76	32.70	110	2.5	54	6.60	94
	44	6.10	32.83	150	1.7	73	6.40	90
	52	6.00	33.44	175	0.9	70	6.39	91
	65	5.61	33.44	240	3.7	81	5.94	84
	87	6.00	34.02	275	2.7	97	5.39	77
	186	6.67	34.90	460	3.7	100	4.69	68
Station 1334 August 4, 1932	1	14.98	32.52	20	0	25	6.10	103
	9	15.02	32.48	27	0	34	6.11	103
	17	14.15	32.66	24	0	38	6.12	102
	26	13.25	32.70	40	1.8	43	6.17	101
	35	12.55	32.70	45	1.9	35	6.24	101
	43	11.86	32.74	60	2.0	37	6.22	99
	52	9.01	32.79	100	2.5	38	6.28	95
Station 1335 August 4, 1932	1	15.50	32.54	25	1.9	26	6.34	108
	10	15.45	32.38	25	2.6	—	6.35	108
	20	12.90	32.74	25	1.4	—	6.68	109
	30	10.61	32.88	65	4.0	71	6.24	97
	40	10.04	32.79	80	4.1	64	6.18	95
	50	10.26	33.01	80	3.9	67	6.18	96
	60	10.11	32.74	85	4.0	62	6.17	95
Station 1336 August 5, 1932	1	15.23	32.70	21	1.0	31	6.25	106
	10	14.35	32.66	22	1.2	32	6.31	106
	20	11.07	32.70	60	3.2	35	6.22	98
	30	10.70	32.66	70	3.9	45	6.12	95
	40	10.65	32.68	70	3.5	37	6.06	94
	50	10.63	32.66	70	3.4	—	6.17	96
	60	10.63	32.74	75	3.9	63	6.10	95
Station 1337 August 5, 1932	1	19.85	32.95	26	1.6	29	5.82	107
	10	17.97	32.63	24	1.1	31	6.12	109
	20	11.49	32.68	24	0.6	28	7.24	114
	30	9.13	32.81	95	7.4	76	5.96	90
	40	8.98	33.44	105	7.4	69	5.95	90
	50	8.97	32.83	105	6.9	77	5.95	90
	60	8.97	32.83	105	7.0	82	5.89	89

Oxygen.—The figures for dissolved oxygen content agree in general with those found in other similar investigations. When the "percentage of saturation" is calculated it is found that the upper 20–40 meters are invariably "supersaturated," with a maximum corresponding to the level of greatest diatom abundance. This is brought out in detail in the section of this report dealing with diatom distribution. In no case is there any clearly defined minimum at an intermediate depth, but always a continuously decreasing percentage to the bottom.

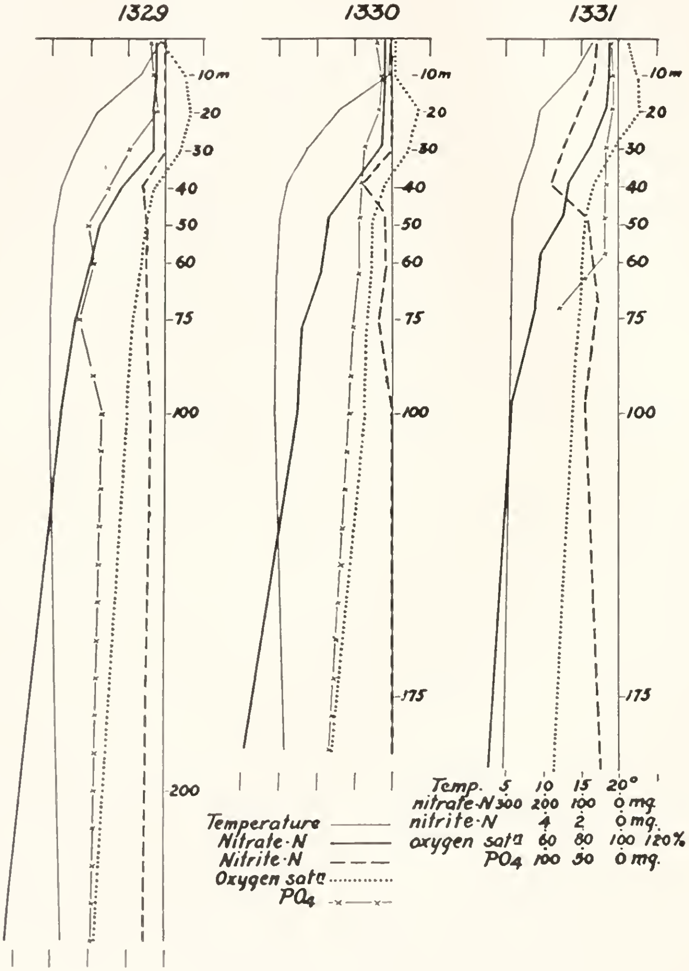

FIG. 2. Chemical data for stations in the deep basin of the Gulf of Maine, August, 1932.

Phosphate.—The phosphate values were obtained under rather less than ideal conditions but are satisfactory for comparative purposes. Only at the first three stations do the concentrations approach critically low values, and it is to be observed that the minimum quantities are found at depths of 10–20 meters, corresponding to the regions of highest oxygen saturation.

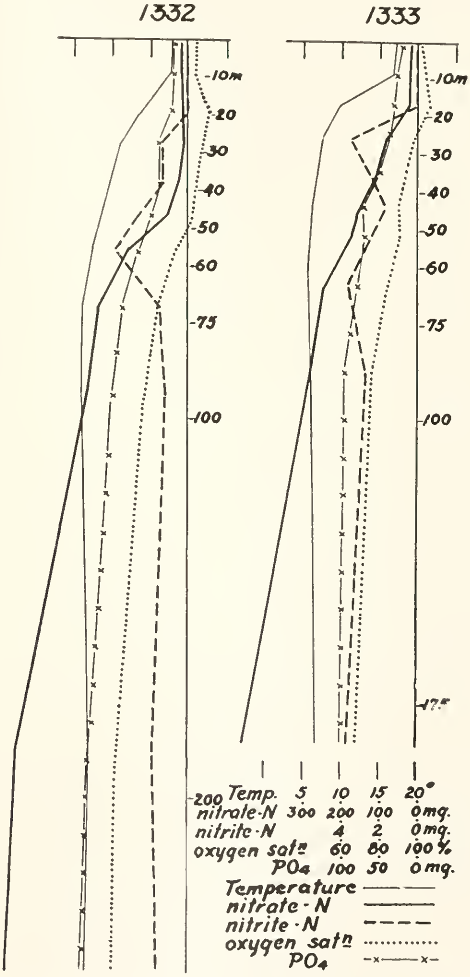

FIG. 3. Chemical data for stations near the Eastern Channel, August, 1932.

Nitrate.—The concentrations of nitrate nitrogen do not in any instance approach what have been considered minimal quantities. However, the correlation of oxygen saturation and diatom abundance with nitrate is much closer than with phosphate. The depth-concentration curves indicate how almost every change in oxygen is accompanied by a similar one in nitrate. The concentrations of nitrate reported here will be observed to be somewhat higher than those generally recorded in the literature. But four years of experience with the method leads the author to believe them fairly reliable. Each sample was determined in duplicate, and the color developed by the reagent compared with a set of dye-standards twice standardized against a series of sea-water samples with known amounts of added nitrate. Deep water samples were diluted with surface water of known nitrate concentration to bring them within the most reliable range of the standards. Anyone who has worked with the method will realize the uncertainties involved in determining absolute amounts of nitrate, although relative values are doubtless satisfactory.

Nitrite.—The nitrite determinations made on this cruise are but one part of a larger study of the variations and general significance of nitrite in the sea, which will be reported later. The position which nitrite occupies in the general nitrogen cycle in the sea has been pointed out on several occasions (9) (10) (7) (11 and others). Its occurrence in sea water, under many circumstances at least, is altogether certain, notwithstanding Harvey's doubts, expressed in 1926 (7). Since it occupies an intermediate position in the oxidation of ammonia to nitrate, its concentration might naturally be expected to be exceedingly variable, depending upon conditions. In fact, there are several agencies, both chemical and biological, which can alter it. The results of Atkins (11) and several others, including the present author, show conclusively that the presence of nitrite is not in any high degree dependent upon prox-

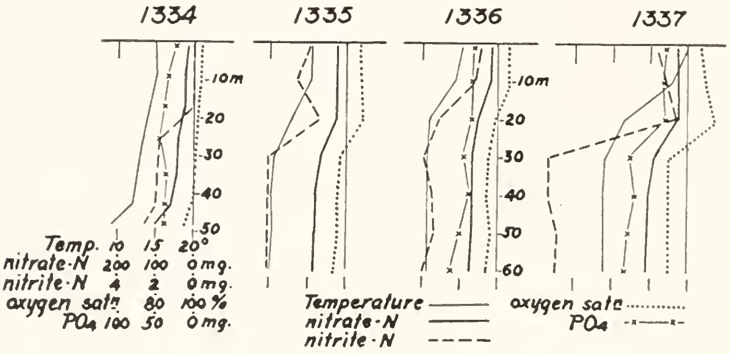

FIG. 4. Chemical data for stations on Georges Bank, August, 1932.

imity to the shore, even in cases in which pollution may be expected. Atkins' assumption (11) as to the significance of the nitrite concentration (namely, that it is an index of the extent of the nitrification process) is hardly adequate. That this is too simple an explanation of its variations may be seen even from the few data here reported, and more particularly on consideration of the bacteriological results in a later section of this report.

Under what may be considered normal conditions at this time of year, the sea water is devoid of nitrite in the upper levels. In only one of the five deep stations, No. 1331, do we find an exception to this, and this station, somewhat isolated in the northern basin of the Gulf, differs from the other stations in further respects. Its thermocline is not so pronounced, and the low phosphate values extend further down. When nitrite is found at the surface it is usually associated with rather large amounts lower down and with evidences of vertical mixing. This can be seen in the depth-concentration curves for Stations 1334–1337.

Another feature of the present investigation, which has been observed by the author on a number of other occasions, is the occurrence of a maximum nitrite concentration somewhere in the neighborhood of 40 meters. The finding of such intermediate maximum values and Waksman's establishment of the fact that the majority, if not all, of the oxidation of ammonia takes place in the bottom, show the inadequacy of regarding nitrite as a mere index of nitrification. Furthermore, Waksman's discovery of nitrate-reducing organisms in the upper levels, and their relation to the diatoms, make it very likely, as will be shown in the third paper of this series, that the nitrite maximum is due to reduction of nitrate rather than to oxidation of ammonia.

The actual bottom water differs in nitrate and nitrite content from that obtained in the lowest sample bottle. The water brought up with sand in the Peterson Grab at Station 1336 was filtered off and found to have a concentration of nitrate nitrogen of 350 mg./m.^{3}, and of nitrite, 25 mg. This may be compared with the nitrate, 75 mg., and nitrite, 3.9 mg., found in the sample at 60 meters, only four meters above. Approximately the same ratio between nitrate and nitrite exists in the two cases, but the enormous difference in the absolute quantities shows clearly where the active nitrification is going on. Again, mud obtained from Station 1331, when washed with nitrite-free surface water, yielded nitrite to the extent of 8.8 mg./m.^{3}, as compared with 1.6 mg. in the lowest water sample. Other similar cases from different areas will be found in a subsequent paper on this general problem.

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STUDIES ON THE BIOLOGY AND CHEMISTRY OF THE GULF OF MAINE

II. DISTRIBUTION OF PHYTOPLANKTON IN AUGUST, 1932

H. H. GRAN[1]

The general distribution of the species of phytoplankton in the Gulf of Maine is well known from Bigelow's papers (1915, 1928). The summer conditions are described (1928, p. 391) as follows:

" In midsummer we have usually found the entire basin of the gulf occupied by a peridinian (Ceratium) plankton, with only occasional diatoms, and we have never found diatoms in abundance anywhere in the gulf in July or August except close along the coast, on the one hand, and on Georges Bank, on the other.

" We found diatoms flowering in abundance on each of our summer visits to the latter locality, but in different regions in different years. Thus they dominated on the western end of the bank on July 9, 1913, and on July 23, 1916, but when we visited that general locality on July 20, 1914, the water contained very few diatoms but, instead, a characteristic peridinian plankton. Three days later, however, we encountered a rich flowering of diatoms near the northeastern edge of the bank. Furthermore, the Georges Bank flowerings of July, 1913 and 1914, though far apart geographically, were both dominated by Guinardia. In July, 1916, however, we found no Guinardia on a traverse of the western part of the bank but swarms of Thalassiothrix and Rhizosolenia in its stead."

. . . " It is not yet clear whether any particular region on the banks is more favorable for the multiplication of diatoms than another, except that we have always found these rich flowerings on its shoaler parts and never close enough to the continental slope to be within the influence of the high temperature outside the edge, which in its own turn, supports various oceanic diatoms in small numbers mingled with peridinians of similarly Tropic origin."

During the cruise with the " Atlantis " in the beginning of August, 1932, we visited the same localities and found on the whole the same general distribution of the communities as in the previous years. The newer methods allowed us to study quantitatively the horizontal and vertical distribution of the various species in correlation with chemical and physical factors, and thus to supplement the previous observations.

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[1] Contribution No. 9, Woods Hole Oceanographic Institution.

At each station water samples for centrifuging were collected from the same water bottles giving samples for chemical determinations. To each sample of 150 cc. were added 5 cc. of a neutralized formalin solution (equal parts of *pure* 40 per cent formalin and distilled water, neutralized by adding drops of a strong sodium carbonate solution to neutral reaction controlled by litmus paper, and carefully filtered before use). Of each sample 55 cc. were centrifuged, and calculated as 50 cc., because experience has showed that about 10 per cent of the content is lost as adhering to the glasses of the centrifuge and to the pipette, even if these are carefully cleaned by rinsing with chromic acid between each operation. The sediments of the four tubes of the centrifuge were carefully transferred to one tube with acute bottom and centrifuged once more. The clear water above the sediment was sucked off cautiously by a pipette and only a sufficient quantity left to be covered by a cover-glass 20 mm. square. The slide is ruled across with 0.4 mm. distance between the lines. For the counting a 4 mm. apochromate (Zeiss) was used. With a compensation ocular No. 4 two intervals are covered by the field of the microscope, with ocular 8 just one (0.4 mm.). The whole content below the cover-glass must be counted, as it is impossible to get the sediments evenly distributed. Every cell is counted also of species forming chains. When one or two chain-forming species are very abundant, it may be sufficient to count the number of chains and calculate the number of cells by multiplication with the average number of cells per chain, separately determined in that same sample. In the tables the frequency of each species is given as cells per liter, the figure of 20 thus representing one cell observed in 50 cc. of sea water.

Vertical hawls with No. 20 silk nets were taken at all stations and used for a preliminary investigation. As a rule all species observed in the net samples were also found in the centrifuged water samples except larger forms as e.g. *Acanthometron*, which was abundant at some of the stations in the deeper part of the Gulf, as also found by Bigelow in 1914. Further a number of nannoplankton species were observed which could not be caught by the nets.

Of the eight stations from which phytoplankton samples were examined, the first five (1329–1333) were taken in the deeper part of the Gulf of Maine, the last three (1334–1336) on Georges Bank. The two groups are best described separately, as they show considerable differences in the quantitative distribution of the species.

The positions of the stations and the data concerning the chemical condition of the sea water are given in the preceding paper (Rakestraw, 1933).

The Deep Basin of the Gulf of Maine

All stations in this area show a marked stratification with regard to the hydrographical conditions as well as to the vertical distribution of the plankton. The surface is covered, downwards to 10 meters or more, by a layer of high temperature, mostly above 15°, and low specific gravity (σ_t less than 25). These surface layers were less saturated with oxygen than the transition layers in the depths of 20–30 meters, which indicates that the photosynthesis of the phytoplankton had been more active at 20

Table I

"Atlantis" Station 1330, August 2, 1932

Depth, *meters*	1	10	19	29	39	48	77
Temperature, ° *C*	19.81	19.43	13.11	8.80	6.03	5.01	4.45
Oxygen, *cc. per liter*	5.56	5.57	6.99	7.26	6.75	6.51	6.34
Oxygen, *% of saturation*	102	102	114	109	96	90	87
Nitrate, *mg. N per cu. m.*	21	21	25	27	105	170	240
	cells per liter	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
Halosphæra viridis juv.	—	—	340	—	—	—	—
Coccolithus pelagicus	220	140	—	—	—	—	—
Pontosphæra Huxleyi	260	220	6640	37200	300	—	—
Dictyocha fibula	—	—	20	80	—	—	—
Actinocyclus Ehrenbergi	—	—	20	—	180	160	120
Corethron hystrix	—	—	—	40	—	20	—
Coscinosira Œstrupi	—	140	—	220	720	160	60
Nitzschia seriata	—	—	40	600	280	—	—
Rhizosolenia alata	40	120	180	160	—	60	—
Rhizosolenia Shrubsolei	—	—	—	40	160	—	—
Ceratium bucephalum	—	—	—	—	140	460	60
Ceratium fusus	20	40	240	360	20	—	—
Ceratium longipes	20	—	20	20	60	280	80
Ceratium tripos	20	—	—	—	—	—	—
Exuviæella baltica	40	100	120	100	—	—	—
Gonyaulax orientalis	20	—	—	40	—	20	—
Gonyaulax orientalis, cystæ	20	40	240	660	40	80	20
Acanthostomella norvegica	—	—	—	20	20	—	—
Labœa conica	20	20	—	—	—	—	—
Labœa strobila	40	60	—	—	—	—	—

The following species were found in small numbers:

Cerataulina Bergonii 30 m., 20. *Chætoceros decipiens* 50 m., 60. *Coscinodiscus centralis* 30 m., 20. *C. excentricus* 30 m., 40. *Leptocylindrus danicus* 30 m., 40. *Nitzschia delicatissima* 30 m., 100. *Thalassiosira decipiens* 40 m., 40. *T. gravida* 30 m., 40.

Oxytoxum gracile 20 m., 20. *Peridinium brevipes* 10 m., 20. *P. Steinii* 10 m., 20. *Protoceratium reticulatum* 30 m., 20.

Favella denticulata 75 m., 20. *Lohmanniella spiralis* 10 m., 20. *Ptychocylis obtusa* 30 m., 20.

Table II

"Atlantis" Station 1331, August 3, 1932

Depth, *meters*	1	10	19	29	39	48	72
Temperature, ° *C*	16.57	13.87	9.65	8.54	6.79	5.81	5.68
σ_t	23.91	24.22	25.49	25.47	25.83	25.96	26.13
Oxygen, *% of saturation*	106	110	111	97	87	83	80
Nitrate, *mg. N per cu. m.*	28	27	35	75	135	150	225
	cells per liter	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
Coccolithus pelagicus	—	—	—	—	20	20	20
Pontosphæra Huxleyi	2060	940	80	—	—	—	—
Distephanus speculum	—	—	—	120	—	40	—
Actinocyclus Ehrenbergi	—	—	—	40	20	20	—
Cerataulina Bergonii	—	—	160	280	—	—	—
Chætoceros borealis	—	—	—	—	160	20	—
Chætoceros compressus	—	—	—	1680	1060*	20*	40*
Chætoceros constrictus	—	—	320	3460	2700*	60	—
Chætoceros decipiens	—	—	—	360	500	40	140
Chætoceros diadema	—	—	—	700	200	—	—
Chætoceros laciniosus	—	—	—	440	160	—	—
Chætoceros radicans	—	—	—	—	80*	—	40*
Corethron hystrix	—	—	—	180	20	—	—
Coscinodiscus excentricus	—	—	—	—	40	20	—
Coscinosira Œstrupi	—	480	60	1800	1520	380	—
Leptocylindrus danicus	—	120	—	120	360	—	—
Rhizosolenia alata	1480	1780	700	200	220	20	20
Rhizosolenia Shrubsolei	—	—	100	200	120	—	—
Thalassiosira gravida	—	—	20	40	20	—	—
Ceratium bucephalum	—	20	260	440	40	—	—
Ceratium fusus	—	20	500	280	—	—	—
Ceratium lineatum	240	—	—	—	—	—	—
Ceratium longipes	—	20	—	80	380	200	40
Ceratium tripos	20	20	280	20	—	—	—
Dinophysis norvegica	20	—	20	—	—	—	—
Exuviæella baltica	—	—	—	—	40	60	120
Gonyaulax orientalis	20	—	280	80	—	—	—
Gonyaulax orientalis, cystæ	—	180	1060	740	100	20	20
Peridinium brevipes	—	—	20	20	20	—	—
Peridinium ovatum	—	—	—	20	80	100	—
Peridinium pellucidum	—	—	—	60	—	20	—
Peridinium Steinii	—	20	120	180	20	—	—
Favella denticulata	—	—	20	20	20	—	—
Lohmanniella oviformis	—	—	20	20	20	—	—
Ptychocylis obtusa	—	—	—	80	60	40	—
Tintinnopsis sp.	—	—	—	80	80	—	—

The following species were observed in small numbers: *Chætoceros affinis* 30 m., 140; *Ch. convolutus* 30 m., 60. *Ch. debilis* 40 m., 360. *Ch. teres*, resting spores 40 m., 80; *Coscinodiscus radiatus* 40 m., 20. *Rhizosolenia fragilissima* 30 m., 160; *Rh. styliformis* 50 m., 20. *Skeletonema costatum* 30 m., 300. *Thalassiothrix longissima* 30 m., 60.

Gonyaulax spinifera 10 m., 20. *Peridinium depressum* 75 m., 40. *P. pyriforme* 30 m., 40. *Acanthostomella norvegica* 20 m., 20.

* with resting spores.

to 30 meters than at the surface. This also agrees with the fact that the phytoplankton had its maximum of frequency just in the same depths, 20 to 30 meters, as shown by Tables I and II, in which the results of the countings are represented for two selected stations, 1330 and 1331.

The dominating species were *Ceratium fusus, Pontosphæra Huxleyi* and germinating cystæ of *Gonyaulax orientalis.* The diatoms were represented by *Rhizosolenia alata,* which also elsewhere as a rule accompanies the Ceratium-plankton during the summer as a subordinate part of the phytoplankton, and by the little *Coscinosira Œstrupi.* Also *Nitzschia seriata* and *Rhizosolenia Shrubsolei* occurred in small numbers. Neritic diatoms were scarce, and some of them had resting spores as a sign that they may be regarded as the last remains of a population from an earlier season. At Station 1331 they were found in greater numbers than elsewhere, with a distinct maximum at 30 meters depth and an accumulation of resting spores at 40 meters. Just at this station the warm surface layer was shallower than at the other stations, the temperature was below 10° from 20 m. and downwards, and the high nitrate content characteristic of the deeper layers was found right up to 30 meters depth (75 mg. N per cu. m.), just where these diatoms had their maximum. Also at this station the surface layer itself was somewhat richer than elsewhere in the Gulf; *Pontosphæra, Rhizosolenia alata,* and *Ceratium lineatum* had their maxima above 10 meters depth.

Table III

Ceratium bucephalum, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	—	—	—	—	—	—	—	20
10	—	—	20	—	—	—	—	—
20	20	—	260	—	20	—	—	—
30	20	40	440	—	—	—	20	—
40	60	140	40	—	—	40	—	—
50	100	460	—	—	—	40	—	—
75 (60)	—	60	—	—	—	—	—	—

At all stations in the Gulf *Ceratium bucephalum* and *longipes,* as shown by Tables III and IV, had their maxima below 30 m., at 40 to 50 m., where the conditions for photosynthesis could not be optimal, judged from the low oxygen content of the water. Only at Station 1331, where the limit between the surface layer and the deeper layers seemed to have been raised to a higher level, *C. bucephalum* had its maximum as high up as 30 m. These two species were dominating the phytoplankton in the same area one month earlier, at the beginning of July, as was observed by the International Passamaquoddy Fisheries Commission, and have probably sunk down after their period of growth.

The two stations 1332 and 1333, taken in the eastern part of the Gulf, where the water according to Bigelow's observations flows into the Gulf from the south, were distinctly poorer than the others. Single specimens of rare oceanic species, like *Oxytoxum reticulatum* at Station 1333, prove the oceanic influence. But at the same time one may be allowed to conclude that the rich Ceratium-plankton of the Gulf has gotten its specific character in the Gulf itself by propagation of the species adapted to the local conditions, even if some of them may have floated in from outside.

GEORGES BANK

The plankton collections taken at the three stations on Georges Bank, 1334 to 1336, all show a marked difference from those of the inner Gulf, but between them there is a great similarity. Table V shows as an example the observations from Station 1334.

TABLE IV

Ceratium longipes, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	—	20	—	—	—	—	—	—
10	—	—	20	—	—	—	—	—
20	—	20	—	—	—	—	40	—
30	—	20	80	—	20	—	20	—
40	60	60	380	—	—	40	—	—
50	—	280	200	—	—	—	20	—
75 (60)	—	80	40	—	200	20	—	—

The species dominating in the Gulf, the *Ceratium* species, *Gonyaulax orientalis, Pontosphæra Huxleyi, Coscinosira Œstrupi,* occurred also on the Bank, but in much smaller numbers. The plankton on the Bank was dominated by oceanic diatoms, first of all *Rhizosolenia alata* (Table VI), which at Station 1334 formed quite a dense population with a maximum of nearly 50,000 cells per liter.

Also, *Rhizosolenia Shrubsolei* and *Nitzschia seriata* occurred in fair numbers, up to 880 and 1440 per liter in nearly all the samples, and *Rhizosolenia styliformis* was found evenly distributed. The neritic diatoms were less abundant; only *Leptocylindrus danicus* and *Thalassiothrix nitzschioides* were found in most of the samples in numbers up to 1400 and 1240 per liter, numbers which are quite inconsiderable in comparison with the quantities of the same species often observed in coastal waters. *Guinardia flaccida,* which was found on the Bank by Bigelow in July, 1913 and 1914 as a dominant species, occurred also in

TABLE V

Atlantis Station 1334, August 4, 1932

Depth, *meters*	1	9	17	26	35	43	52
Temperature, ° *C*	14.98	15.02	14.15	13.25	12.55	11.86	9.01
σ_t	24.08	24.04	24.37	24.58	24.73	24.88	25.41
Oxygen, *% of saturation*	103	103	102	101	101	99	95
Nitrate, *mg. N per cu. m*	20	27	24	40	45	60	100
	cells per liter	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
Coccolithus pelagicus	—	—	—	—	—	40	20
Pontosphæra Huxleyi	840	160	—	—	—	—	—
Dictyocha fibula	—	40	—	—	—	—	20
Actinocyclus Ehrenbergi	—	—	80	80	40	—	120
Coscinodiscus excentricus	—	—	40	80	—	—	40
Coscinosira Œstrupi	40	—	—	120	—	80	140
Guinardia flaccida	—	—	—	—	—	40	100
Leptocylindrus danicus	—	—	1240	320	480	880	160
Nitzschia delicatissima	—	160	240	400	200	480	40
N. seriata	120	680	560	1080	1160	1440	1060
Paralia sulcata	—	—	—	—	—	—	500
Pleurosigma Normani	—	40	200	80	40	40	60
Rhizosolenia alata	27880	29720	47920	49800	27360	30120	15860
R. Shrubsolei	560	120	400	880	760	200	460
R. styliformis	200	120	40	120	120	40	140
Skeletonema costatum	—	—	—	160	—	160	—
Thalassiothrix longissima	80	—	40	40	160	—	—
T. nitzschioides	280	440	1240	960	560	1200	860
Ceratium bucephalum	—	—	—	—	40	40	—
C. fusus	—	120	80	—	40	—	100
C. lineatum	—	40	—	—	80	—	60
C. longipes	—	—	—	—	40	—	20
C. tripos	40	—	—	—	—	—	20
Gonyaulax orientalis, cystæ	40	—	—	—	—	40	20
Favella denticulata	—	—	80	40	40	40	40
Helicostomella subulata	40	40	40	80	120	40	40

The following species were observed in small numbers:

Actinoptychus undulatus 10 m., 40; *Chætoceros compressus* 20 m., 320; *Leptocylindrus minimus* 20 m., 640; *Rhizosolenia setigera* 50 m., 80; *Dinophysis acuminata* 20 m., 40; *Peridinium brevipes* 50 m., 40; *Pyrophacus horologium* 40 m., 40.

Ptychocylis obtusa 50 m., 20; *Lohmanniella spiralis* 60 m., 20; *Tintinnopsis* sp. 60 m., 20.

1932 at all three stations, but only in the samples from the deeper layers (Table XIV). It was most abundant at Station 1336 with a maximum of 340 per liter in 50 meters depth. The neritic tintinnid *Helicostomella subulata* was found evenly distributed at all three stations in numbers up to 120 per liter.

Very characteristic is the regular occurrence of some littoral diatoms, *Pleurosigma Normani*, *Paralia sulcata*, and *Navicula distans*. Their distribution indicates with certainty turbulence of the waters by which material from a shallow bottom has been carried out to sea.

Table VI

Rhizosolenia alata, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	160	40	1480	—	80	27880	3080	440
10	220	120	1780	120	20	29720	4320	1750
20	100	180	700	280	20	47920	23620	4040
30	60	160	200	220	20	49800	12720	1660
40	140	—	220	120	40	27360	7660	2720
50	—	60	20	20	20	30120	8220	1720
75 (60)	—	—	20	—	—	15860	5720	660

Table VII

Pleurosigma Normani, August 4–5, 1932

Depth	Stations		
	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*
1	—	—	20
10	40	20	25
20	200	20	120
30	80	40	60
40	40	120	160
50	40	40	60
60	60	40	220

It is also remarkable that on the Bank all the species mentioned above were evenly distributed from the surface to the bottom (Tables VI and VII). Such a situation can only have been produced by a strong vertical mixing of the waters, which is also indicated by the even distribution of the temperature and salinity. The diatoms show a slight maximum at all stations at the depths of 20 to 30 meters. Just at this depth the photosynthesis seemed to have its optimum in the Gulf and the same may have been the case on the Bank, and thereby the growth may have been stimulated. But the difference in numbers corresponds only to one or two cell divisions and thus the turbulence of the waters must have had a stronger influence on the vertical distribution of the diatoms than the factors regulating their growth. The higher temperature of the very surface layer, from 1 to 10 meters, seems to have been slightly unfavorable for the growth or perhaps for the floating power—because of the lower viscosity of the water—of most of the species, for instance *Leptocylindrus danicus* (Table VIII).

TABLE VIII

Leptocylindrus danicus, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	—	—	—	—	—	—	—	—
10	—	—	120	—	—	—	—	—
20	—	—	—	—	20	1240	1400	560
30	—	40	120	—	—	320	1340	440
40	—	—	360	—	—	480	580	420
50	—	—	—	—	—	880	300	380
75 (60)	—	—	—	—	—	160	640	240

A comparison between the Gulf and the Bank shows first of all that the same species are found over the whole area, but the quantitative and also the vertical distribution is different. The stratification in the inner Gulf has favoured the growth of the *Ceratia* and of *Pontosphæra* (Tables IX and X), accumulated by their active movements in the depths of optimal life conditions. On the other hand, the mixing of the waters on Georges Bank has brought into circulation sufficient quantities of nutrient salts to produce a strong growth of oceanic diatoms, *Rhizosolenia alata* and others, and the continued vertical mixing has caused their even distribution from surface to bottom. At the innermost station on the Bank, Station 1334, this population has reached a nearly maximal density for so large a species (Tables V and VI).

Neritic diatoms, which are as far as we know, the most rapid growers of the plankton of temperate seas, were quite scarce over the whole area, although single cells or chains were observed everywhere. The only locality where a slight accumulation of them could be observed, was Station 1331, where for some unknown reason the nitrate content was high right up to 30 meters depth, and just at this depth the diatoms had increased (Table II). Evidently they require a higher concentration of nutrient salts than the surface layers of the Gulf of Maine contained at this season.

TABLE IX

Ceratium fusus, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	80	20	—	—	—	—	—	40
10	220	40	20	20	60	120	40	20
20	420	240	500	40	40	80	100	40
30	620	360	280	—	—	—	—	20
40	—	20	—	—	—	40	—	—
50	—	—	—	—	—	—	—	—
75 (60)	—	—	—	—	—	100	—	—

TABLE X

Pontosphæra Huxleyi, August 2–5, 1932

Depth	Stations							
	1329	1330	1331	1332	1333	1334	1335	1336
meters	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*	*cells per liter*
1	240	260	2060	260	2720	840	40	—
10	3880	220	940	960	2120	120	80	80
20	15620	6640	80	860	10660	—	2680	—
30	2000	37200	—	580	4140	—	40	—
40	80	300	—	40	3060	—	20	20
50	—	—	—	40	80	—	—	—
75 (60)	—	—	—	—	—	—	—	60

CULTURE EXPERIMENTS

As a contribution to the question of the requirements of nutrition of the plankton diatoms some culture experiments were made with the same method as was used previously on the Norwegian coasts. A series of closed, glass-stoppered bottles containing sea water with known content of plankton were suspended in the sea, all under the same conditions of light and temperature, and with the addition of various nutrient salts. The increase of the diatoms was determined by counting. When the culture bottles were exposed only a few days, and the population in the bottles was not allowed to increase above the maximum density observed in the sea, about one million cells per liter, the cultures could be kept under quite normal conditions, and by use of a hand lens the cells or chains could be seen normally and evenly suspended in the water within the bottles.

The main question for the experiments arranged at Woods Hole was that of the possible influence of soluble iron compounds on the growth of the plankton diatoms. As it is now well known, most of the differences in the productivity of the various areas in the sea can be explained from the distribution of the nitrates and phosphates, acting as limiting factors for the growth of the plankton algæ according to Brandt's theory.

But we have also some indications that the quantities of soluble iron present in sea water may be insufficient to support a rapid growth, particularly of the neritic diatoms, and my working hypothesis is that the soluble iron compounds, washed out with "humus"-compounds from the shores, may give the explanation of the relative productivity of coastal waters in comparison with the open ocean.

In an experiment made on the Norwegian coast (Gran, 1931) I had found that the growth of the neritic diatom *Skeletonema costatum* was enormously stimulated by the addition of small quantities of garden soil extract, corresponding to 10 mg. soluble Fe per cu. m., to the oceanic water in which it was living. In this water, which contained sufficient quantities of nitrates and phosphates, no other nutrients gave, when added, any positive effect at all.

If the effect of the soil extract is caused by its content of soluble iron, it must be possible to replace the soil extract by other soluble iron compounds, as Carsten Olsen (1929) did in his experiments with *Lemna*, using ferri-citrate with positive results. Recently also Burk and his collaborators (Burk, Lineweaver, and Horner, 1932) have proved by exact experiments that the stimulating effect of soil extract on *Azotobacter* is caused by its content of soluble iron.

My previous experiments to replace the soil extract by other iron compounds were not very successful. Oxyhæmoglobin or ferrisaccharate gave no positive effect, and the effect of ferricitrate or ferrosulphate was too small in comparison with that of the soil extract, possibly because these compounds were too quickly decomposed or oxidized in sea water.

At Woods Hole Dr. Waksman kindly gave me an opportunity to test his synthetic ferri-ligno-proteid (Waksman and Iyer, 1932) which, according to his great experience, may be regarded as identical with the essential components of "humus" and which at least has the same biological and chemical reactions.

For the experiments clean, glass-stoppered bottles of 130 cc. inner volume were filled from the same container with sea water of known plankton content, and suspended in the harbor at a depth of 1.5 meters.

To the bottles was added:

(1) No addition (3 bottles).
(2) 1 cc. of a solution containing 0.1 per cent KNO_3 and 0.01 per cent NaH_2 PO_4 (3 bottles).
(3) The same as (2) + 1 cc. of garden soil extract (1 liter garden soil, boiled in autoclave with 1 liter distilled water, then filtered, and the filtrate sterilized in autoclave) (3 bottles).
(4) The same as (2) + 0.1 cc. of a solution of 100 mg. ferri-ligno-protein in 500 grams water (3 bottles).

Table XI

Culture Experiment 1. Woods Hole, July 23–26. Temperature, 20°–22°. Surface water taken outside the harbor.

Diatoms	Before experiment	Without addition (1)	Nitrate and Phosphate (2)	Nitrate, Phosphate, and Soil (3)	Nitrate, Phosphate, and Ferri-ligno-protein (4)
	cells per cc.	*cells per cc.*	*cells per cc.*	*cells per cc.*	*cells per cc.*
Chætoceros compressus	2	1	243	310	415
Leptocylindrus danicus	13	16	466	792	628
Nitzschia delicatissima	28	58	957	595	789

The first experiment was made on July 23 with surface water taken outside the harbor of Woods Hole. This water was rather rich in diatoms, and therefore the bottles were suspended for only three days, until July 26, and their content preserved with formalin for counting. The water originally contained per cubic centimeter 22 cells of *Rhizosolenia calcar avis*, 24 of *R. Shrubsolei*, 2 *Chætoceros compressus*, 13 *Leptocylindrus danicus*, 28 *Nitzschia delicatissima* and some other species in very small numbers. The two first-named species did not grow well in the bottles; for the other three the average number per cubic centimeter is given in Table XI.

The result is clear. Without addition of nutrients the diatoms had not propagated at all, but the addition of nitrate and phosphate, in quantities found under optimal conditions in nature, was enough to stimulate them to a growth about equal to the maximal growth observed for plankton diatoms, corresponding to seven divisions in three days for *Chætoceros compressus* and five to six divisions for the other species. The extra addition of soil extract or of the ferri-ligno-protein had no marked effect. If the stimulating effect of the soil extract depends upon its iron content, the conclusion must be that the harbor water used for the experiment contained more than enough of soluble iron, while at the same time its content of nitrate or phosphate has been the limiting factor for further growth of the diatoms.

The experiment needed to be repeated with more oceanic water which, according to the theory, should contain less soluble iron if these compounds are renewed by outwash from shore. Therefore, on the last night of the cruise of the "Atlantis," the fifth of August, a large water sample was taken from the surface off Nantucket Lightship and a new experiment arranged the next day. The same plan as before was followed, except that to the bottles of series (4) was also added $MnCl_2$, corresponding to 10 mg. Mn per cubic meter. The water was rather poor in plankton. It contained six cells per cubic centimeter of *Rhizosolenia alata* and 0.4 cells per cubic centimeter of *Nitzschia seriata*. *Leptocylindrus danicus* must have been present to the number of 0.1 cells per cubic centimeter, although it was not found by counting a sample of 10 cc. The character of the plankton was rather similar to what was found at Station 1336 on Georges Bank.

The species reacted in different ways to the addition of the nutrients. *Rhizosolenia alata*, which is an oceanic species, had in five days without extra nutrition grown to make up a population of about the same density which was found at the innermost station on Georges Bank, Station 1334. With nitrate and phosphate it had grown very much more. The increase corresponds to six cell divisions in five days, which I have found to be the maximal growth rate for this species. The extra addition of soil extract or ferri-ligno-protein did not give any further effect.

The typical neritic species, *Leptocylindrus danicus,* reacted otherwise. With addition of nitrate and phosphate alone it increased from one to 130, which corresponds to seven divisions in five days, one cell division more than *Rhizosolenia*. But with soil extract it increased to a number ten to eleven times higher, and with Waksman's compound and $MnCl_2$ twenty-five fold. With soil extract it averaged ten to eleven cell divisions in five days, and with ferri-ligno-protein eleven to twelve divisions. The effect of the synthetic "humus" seems to be quite similar to that of the soil extract.

Nitzschia seriata also showed some effect of the soil extract and of the ferri-ligno-protein, but less marked than *Leptocylindrus*. In its distribution it is not so strongly limited to the coastal waters as *Leptocylindrus*. It is one of the very few motile species among the plankton diatoms, which may perhaps give the explanation of its relatively good growth in the bottles without added nutrition.

We intended to continue the experiments and to use for comparison also ligno-protein without iron, but had no opportunity to get water of sufficiently oceanic character. A third experiment gave results parallel to those of the first, a maximal effect of nitrate and phosphate alone and no extra effect of the iron compounds.

The results published here are not conclusive and the experiments should be repeated when opportunity arises. The method is practicable where water with living plankton can be brought in from oceanic situations.

The question, whether the presence of soluble iron compounds in sufficient quantities can be a limiting factor to the growth of the plankton diatoms, and particularly of the species with the most rapid growth rates, can not be solved, of course, until we also have more data about the distribution of the soluble iron compounds in the sea.

TABLE XII

Culture Experiment 2. Woods Hole, August 6–11, 1932. Temperature 20–22°. Water taken off Nantucket Lightship on the night of August 5.

Diatoms	Before experiment	Without addition (1)	Nitrate, Phosphate (2)	Nitrate, Phosphate, and Soil (3)	Nitrate, Phosphate, ferriligno-protein, and Manganese (4)
	cells per cc.	*cells per cc.*	*cells per cc.*	*cells per cc.*	*cells per cc.*
Rhizosolenia alata	6	29	403	466	478
Nitzschia seriata.	0.4	36	365	1109	769
Leptocylindrus danicus	0.1	1	13	150	335

The diatoms were quite normal in all cultures; in some bottles they had even formed auxospores. The figures represent averages of three parallel cultures.

In collaboration with Dr. Waksman and Dr. Reuszer a preliminary experiment was also arranged to follow the relation of the marine bacteria to the plankton diatoms.

A series of bottles filled with sea water taken at the surface outside the harbor of Woods Hole were suspended in the sea in the same way as described above. To some of them was added nitrate and phosphate in the same relation as in Experiments 1 and 2, while the others were left without any addition of nutrients. Bottles were taken to be studied with regard to their content of plankton and bacteria after three, seven and twelve days. The water was rather rich in plankton beforehand, and the number did not increase in the bottles without nitrate and phosphate. As shown in Table XIII, the plankton in the fertilized bottles had increased after three days to a maximal density. Most of the species were still in good condition and normally suspended in the water. After seven days most of the species had decreased in number, and dead cells formed lumps at the bottom of the bottles.

The bacteria increased and decreased with the diatoms, as Waksman and his collaborators report (in the paper following this one). The high temperature may have been one of the causes accelerating the decrease of the plankton after the maximum. Waksman's interesting idea of the correlation between the plankton and the bacteria can perhaps be studied favorably in experiments like this, in which the water is poor in plankton from the beginning and therefore the conditions in the bottles can be kept normal for some days. When some of the diatoms begin to die too many unknown factors are introduced.

Table XIII

Culture Experiment 4. August 18–30, 1932. Nitrate and phosphate as in Experiment 1 (2).

Diatoms	Before experiment	After 3 days	7 days	12 days
	cells per cc.	*cells per cc.*	*cells per cc.*	*cells per cc.*
Chætoceros compressus	16	95	18	57
Coscinosira Œstrupi	11	62	6	3
Leptocylindrus danicus	11	86	5	0
Nitzschia seriata	65	468	65	31
Rhizosolenia calcar avis	2	0	0	0
Rhizosolenia delicatula	36	32	7	0
Rhizosolenia fragilissima	21	84	37	0
Rhizosolenia setigera	6	6	21	27
Rhizosolenia Shrubsolei	1	0	0	0
Skeletonema costatum	(0)	139	3	9
Thalassiothrix nitzschioides	2	16	0	0
Other species	6	38	16	38
Sum	177	1026	178	165

Remarks on the Species

Heterocontæ

Halosphæra viridis Schmitz is the only annual plant of the phytoplankton, as far as known. It has never been observed to occur in such an abundance as to be found in great numbers in the centrifuged water samples. Young specimens were found at Station 1330 at 20 meters depth in a number of 340 per liter, and at Station 1333 it was observed in all samples from the surface to 50 meters, with a maximum of 140 per liter at 20 meters. Single specimens were observed at Stations 1332, 1334, and 1336.

Coccolithophorida

Coccolithus pelagicus Wallich was observed at the three stations in the inner part of the Gulf (1329–1331) and at Station 1336 on Georges Bank. Its maximum of frequency, 220 cells per liter, was found at Station 1330 near the surface, 1–10 meters. At Station 1336 it occurred in all samples from 20 meters downwards, with a maximum of 100 at 40 meters depth.

Pontosphæra Huxleyi Lohmann is the most common of all the Coccolithophorida. Although it is an oceanic species it can occur in enormous numbers, millions per liter, in coastal waters also, where the temperature is sufficiently high. As the table shows, it was found over the whole area, distinctly more abundant at the deeper stations in the Gulf than on Georges Bank, with a maximum of frequency of 37,000 per liter at Station 1330.

Silicoflagellata

The few species of this order have a wide distribution but never occur in abundance.

Dictyocha fibula Ehrenberg was observed at two stations in the Gulf and at three stations over Georges Bank, nowhere in higher numbers than 80 per liter.

Distephanus speculum Ehrenberg is a more northern species, which was observed only at Station 1331, together with the maximum of neritic diatoms, most abundant (120 per liter) at 30 meters depth, and at Station 1336.

Diatoms

In temperate and arctic waters the diatoms make up the bulk of the phytoplankton where the nutrient salts are present in sufficient quantities. Some of them, particularly the neritic species, as *Skeletonema costatum, Chætoceros debilis, C. compressus,* and others, *Leptocylindrus danicus, Thalassiosira Nordenskiœldii,* are able to reproduce themselves under favorable conditions very quickly, with up to two cell divisions per day. Also some oceanic species, as *Rhizosolenia alata* and *R. semispina, Chætoceros decipiens, Nitzschia seriata,* and *N. delicatissima* can form very dense populations, but their propagation seems to be less rapid than that of the neritic ones mentioned above. Other species as, e.g., the large *Coscinodiscus,* can be very commonly distributed but never so abundant that more than a few specimens are found by centrifuging

50 cc. of sea water. A number of littoral species occur so regularly in the plankton that they have to be reckoned with as signs of turbulence of the water, although their number is quite insignificant in comparison with the true pelagic species. It is rather remarkable that only some few out of the great number of littoral species are found regularly in the plankton, as in our material *Actinoptychus undulatus, Navicula distans, Pleurosigma Normani,* and *Paralia sulcata.* They may perhaps even propagate to some extent in the floating condition.

Actinocyclus Ehrenbergii Ralfs. This species was observed at three stations in the central part of the Gulf (1329–1331) and at all stations on Georges Bank (1334–1336). Here it was found in all depths from 20 meters to the bottom in numbers of up to 300 per liter at Station 1336, at 30 meters depth. Its heavy, thick-walled cells are more often found in greater depths than in the surface layers, and it is in my opinion a characteristic representative of the " shade-flora " of Schimper (Karsten, 1905). Ostenfeld (1913) regarded it as a littoral, " tychopelagic " species.

Actinoptychus undulatus Ehrenberg was observed as single specimens at all stations on Georges Bank. It is without doubt a littoral species.

Cerataulina Bergonii H. Peragallo. This neritic species had a small maximum in 20 to 30 meters depth at Station 1331 (160 to 180 per liter) together with the neritic *Chætoceros* species. Single specimens were also observed at Stations 1330, 1332, and 1336.

Chætoceros. Eleven species of this genus were observed. All of them except the oceanic species *Ch. atlanticus* occurred and had their maximum at Station 1331 (see Table II) at 30 to 40 meters depth as remains of a more abundant growth earlier in the season. At 30 meters the high nitrate content of the water may have caused some increase, but at 40 meters many of the species had formed resting spores and were evidently sinking. At Station 1329 the situation was similar but less pronounced. Here only the two most common species, *Ch. compressus* and *Ch. constrictus* occurred in small numbers (120 to 260 per liter), the former with resting spores.

On Georges Bank the oceanic species *Ch. atlanticus* and particularly *Ch. decipiens* occurred at the two outer stations, 1335 and 1336, *Ch. atlanticus* very scarce in a few samples (60–80 per liter), *Ch. decipiens* in all samples except at the surface, in numbers up to 340 per liter at 20–30 meters at Station 1335. Here also the neritic species *Ch. compressus* and *laciniosus* were found in small numbers, mostly with resting spores.

The species found in the material were: *Ch. affinis* Lauder, *Ch. at-*

lanticus Cleve, *Ch. borealis* Bailey, *Ch. compressus* Lauder, *Ch. constrictus* Gran, *Ch. convolutus* Castracane, *Ch. debilis* Cleve, *Ch. decipiens* Cleve, *Ch. diadema* Ehrenberg, *Ch. laciniosus* Schuett, *Ch. radicans* Schuett, *Ch. teres* Cleve.

Corethron hystrix Hensen occurred in small numbers at Stations 1330 and 1331, with a maximum of 180 per liter at 30 meters depth at Station 1331.

Coscinodiscus. The centrifuge method cannot give a good picture of the distribution of the larger species of this genus, as the quantity of water (50 cc.) is too small. *C. centralis* Ehrenberg was observed at Station 1330, *C. radiatus* Ehrenberg at Stations 1331 and 1336, but only as single specimens. *C. excentricus* Ehrenberg was found more regularly, at all stations except 1329 and 1332. It seemed to be more common over Georges Bank than in the deeper parts of the Gulf, with maximum records of 80 per liter at 30 meters depth at Stations 1334 and 1336.

Coscinosira Œstrupi Ostenfeld occurred regularly at all stations, less abundant on Georges Bank than in the inner part of the Gulf, and with a maximum of abundance 1800 per liter at Station 1331, at 30 meters depth, together with the maximum of the neritic *Chætoceros* species. This little species of which Hustedt (1927–30, p. 317) recently has given excellent figures and description, is one of the very few representatives of the plankton of the western side of the North Atlantic, which is lacking, or at least very rare along the European coasts.

Guinardia flaccida H. Peragallo was dominating in the plankton of Georges Bank in July, 1913 and 1914 (Bigelow, 1928). It occurred also in August 1932 at all stations on the Bank, but not in great numbers, and with its abundance increasing toward the bottom, as shown by Table XIV. *Guinardia* was not observed in the deeper parts of the Gulf.

Leptocylindrus danicus Cleve (see Table VIII) occurred regularly at all stations on Georges Bank from 20 meters to the bottom, with maximum at 20 meters depth at all three stations. It was also found in small numbers at Station 1331 together with the *Chætoceros*-plankton and in traces at Stations 1330 and 1333.

Leptocylindrus minimus Gran was observed in small numbers, up to 640 per liter at Stations 1334–1336 on Georges Bank, at 20–30 meters depth.

Navicula distans W. Smith was observed as single specimens at Stations 1329 and 1335. At Station 1336 on Georges Bank it was found in all samples from 10 meters to the bottom. It is a littoral species with thick cell walls.

Nitzschia closterium W. Smith. Single specimens at Stations 1329 and 1336.

Nitzschia delicatissima Cleve occurred regularly at all depths from 20 meters to the bottom at Stations 1334–1336 on Georges Bank, but in very small numbers, up to 480 per liter. At Stations 1329 and 1330 traces were observed.

Nitzschia seriata Cleve occurred as well in the inner part of the Gulf as on Georges Bank, with a maximum of 1440 per liter at Station 1334.

Paralia sulcata (Ehrenberg) is another littoral species, which was found as scattered chains at Stations 1332–1336 in the deeper layers of water.

Pleurosigma Normani Ralfs was observed in nearly every sample from Georges Bank, rather evenly distributed from the surface to the bottom, as a sign of the turbulence. In a sample of 50 cc., from one to eleven cells were counted (Table VII).

TABLE XIV

Guinardia flaccida, August 4–5, 1932

Stations.......		1334	1335	1336
		cells per liter	*cells per liter*	*cells per liter*
Depth in meters	1	—	—	—
	10	—	—	—
	20	—	—	20
	30	—	—	160
	40	—	20	40
	50	40	140	300
	60	100	220	340

Rhizosolenia alata Brightwell was, in accordance with Bigelow's observations in previous years, the most common diatom at this season over the whole area and occurred at all stations, subordinate to the *Ceratia* in the stratified waters in the deeper part of the Gulf, but dominant on Georges Bank. Here it was distributed rather evenly from surface to bottom with a slight maximum at 20 to 30 meters depth. At Station 1334 at the inner side of the Bank the population was quite dense, with a maximum of nearly 50,000 cells per liter.

Rhizosolenia fragilissima Bergon (including *R. færoënsis* Ostenfeld), a neritic species which sometimes occurs in abundance, was observed only at Station 1331 at 30 meters depth in quite small numbers, together with the maximum of neritic *Chætoceros* species.

Rhizosolenia Shrubsolei Brightwell occurred together with *R. alata*, as it often does, but in much smaller numbers. On Georges Bank it was found evenly distributed from the surface to the bottom, in numbers up to 800–900 per liter. In the deeper part of the Gulf it was much less frequent and was limited to the layers between 20 and 40 meters depth.

Rhizosolenia styliformis Brightwell. This large and beautiful oceanic diatom had the same distribution as *Rh. Shrubsolei*, but occurred in smaller numbers. The highest frequency observed was 520 per liter at Station 1335 at 60 meters depth, but as a rule the number counted was only 200 per liter or less.

Rhizosolenia setigera Brightwell is a neritic species which was observed only in quite small numbers on Georges Bank, less than 100 per liter.

Skeletonema costatum (Greville). Single chains were observed at Stations 1331 and 1334.

Thalassiosira decipiens (Grunow) was observed quite rarely between 40 and 50 meters depth at the first two stations (1329–1330).

Thalassiosira gravida Cleve occurred at most of the stations but was quite scarce. The highest number observed was 140 per liter at Station 1329, 50 meters depth.

Thalassiothrix longissima Cleve and Grunow occurred rather evenly on Georges Bank, particularly at Station 1334, where it reached a maximum of 160 per liter at 40 meters. It is regarded as a subarctic oceanic species.

Thalassiothrix nitzschioides Grunow was only observed on Georges Bank but occurred there evenly distributed from surface to bottom in numbers from 200 to 1200 cells per liter.

Dinoflagellata

Ceratium. The species of this genus are by far the most important among the Dinoflagellata of the North Atlantic. They are oceanic, but are often found in greater abundance in semi-coastal areas as the North Sea, the Gulf of Maine, and the Gulf of St. Lawrence than in pure oceanic waters. Here they usually begin to dominate the phytoplankton as soon as the waters become stratified, after the spring growth of the diatoms has finished. They succeed each other according to their temperature requirements. *C. arcticum* and *C. longipes* dominate in the first part of the season, followed by *C. fusus* and *tripos*.

In the Gulf of Maine *C. fusus* was the dominating species in the beginning of August (Table IX) in the stratified waters. Its maximum of 500–600 per liter was found just at the depth where the values for saturation with oxygen were maximal and the life conditions optimal for the phytoplankton, at 20 to 30 meters depth. Two other species, *C. longipes* and *C. bucephalum*, were also rather abundant at the same stations (Tables III and IV) but their maxima were found deeper down, 40–50 meters depth, evidently below the depth of the optimal conditions for photosynthesis, as the water at these depths was not saturated with oxygen. Other observations, from the investigations of the International Passamaquoddy Fisheries Commission, show that these two species were dominating not far from the localities where our stations were taken, one month earlier, in the beginning of July. One may conclude that they have sunk into deeper layers after their main growth period was ended and they were succeeded by *C. fusus*. From my experiences on European coasts it is astonishing to find *C. bucephalum* early in the season together with *C. longipes,* as we used to find its maximum in September, together with that of *C. furca* which I have not observed at all in the Gulf of Maine.

Besides these species *C. arcticum* was found in small numbers at Station 1329, from 20 to 50 meters depth.

Ceratium tripos (O. F. Mueller) occurred at the same stations as *C. fusus*, and at the same depths, but less abundantly. Its maximum was found at 20 meters at Station 1331, with 280 per liter.

Ceratium lineatum (Ehrenberg) seems to be more neritic than the other species. It was found at the surface at Station 1331 in the number of 240 per liter, and on Georges Bank it occurred at all stations, rather evenly distributed with regard to the depth, with a maximum of 150 per liter at Station 1336, at 10 meters depth.

Dinophysis acuminata (Claparède and Lachmann) was observed as single specimens at two stations, 1329 and 1334.

Dinophysis norvegica Claparède and Lachmann was also quite scarce. Single cells were observed at four stations, in seven samples.

Exuviælla baltica Lohmann occurred at all stations with its maximum near the surface. The highest number counted was 480 per liter at 10 meters depth at Station 1335. In coastal waters it can sometimes be found in much greater numbers.

Gonyaulax orientalis Lindemann. This species, described also by Miss Lebour (1925, p. 93) seems to be rather common. Rather few vegetative cells were found, but a number of globular thick-walled cysts, containing chromatophores and oil drops. Some of them were in cell division, and two naked cells escaped which developed into *Gonyaulax* cells. They were rather abundant in the inner part of the Gulf in depths from 10 to 30 meters, with a maximum of 1340 at Station 1331, but less common on Georges Bank. The diameter of the cysts was from 45 to 64 μ, the thickness of their cell wall 1.5 μ.

Gonyaulax spinifera (Claparède and Lachmann) was rare in the deeper part of the Gulf. At Station 1329 80 cells per liter were found as a maximum.

Oxytoxum gracile Gran (1929, p. 45). Single specimens were observed at two stations (1330 and 1332).

Oxytoxum reticulatum (Stein). One specimen of this rare oceanic species was observed at Station 1332 at 20 meters depth.

Peridinium

The marine species of *Peridinium* (exclusive of *Minuscula* Lebour) are heterotrophic and rarely occur abundantly enough to be regularly found in the centrifuged water samples. Eight species were observed: *P. achromaticum* Levander, *P. conicum* Gran, *P. brevipes* Kofoid, *P. depressum* Bailey, *P. ovatum* Pouchet, *P. pellucidum* Bergh, *P. pyriforme* Paulsen, *P. Steinii* Joergensen. Of these only *P. Steinii* and *P. ovatum* were found in a maximum abundance of 100 per liter or more, both of them at Station 1331, where seven of the eight species were found.

Protoceratium reticulatum Bergh, one specimen at Station 1329.

Pyrophacus horologium Stein. This neritic and autotrophic species was found at all stations on Georges Bank, but always in small numbers, up to 60 per liter.

Infusoria ciliata

Acanthostomella norvegica (Daday) was observed in small numbers at four stations in the deeper part of the Gulf, with the maximum of 80 per liter, at Station 1329, 20 meters depth.

Favella denticulata (Ehrenberg), the most common tintinnid in the collections, was found at all stations, mostly in 20 to 30 meters depth, with a maximum of 320 per liter at Station 1329, 20 meters depth.

Helicostomella subulata (Ehrenberg) is a neritic species which was found exclusively on Georges Bank, and here at all stations, with the maximum of 220 per liter at Station 1335, 20 meters depth.

Labœa Lohmann. The species of *Labœa* are not found in the net samples, but some of them which live in symbiosis with small brown cells can at times occur rather abundantly in the centrifuged water samples, particularly in spring together with the neritic diatom plankton. In August they were very scarce. *L. conica* Lohmann and *L. strobila* Lohmann were observed at Station 1330 from 1 to 10 meters depth, *L. acuminata* Leegaard and *L. vestita* Leegaard at Station 1335.

Lohmanniella oviformis Leegaard was observed in small numbers at four stations, 1331–1333 and 1336. At Station 1333 it occurred from 10 to 30 meters depth with a maximum of 60 per liter at 30 meters.

Lohmanniella spiralis Leegaard. Single specimens were observed at Stations 1330 and 1334.

Mesodinium rubrum Lohmann was quite scarce at this season. At Station 1335 on Georges Bank about 100 per liter were found at 1 to 10 meters depth, at Station 1333 a single specimen at 20 meters.

Ptychocylis obtusa Brandt was observed at Stations 1329–1331 in the Gulf and on Georges Bank at Stations 1334 and 1335, with a maximum of 80 per liter at Station 1331, 30 meters depth. A single specimen of *Tintinnopsis campanula* (Ehrenberg) was found at Station 1335.

Stenosemella sp. occurred on Georges Bank and at Stations 1332–1333 with a maximum of 100 per liter at Station 1335, 20 meters depth.

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STUDIES ON THE BIOLOGY AND CHEMISTRY OF THE GULF OF MAINE

III. Bacteriological Investigations of the Sea Water and Marine Bottoms

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In former expeditions undertaken for the study of marine life, the occurrence, abundance, and activities of the bacteria were either left out entirely, or were based upon the examination of samples of water brought back many days and frequently months after they were collected. The bacteriological activities in the sea bottom were frequently completely overlooked. In view of the fact that the total numbers of the bacteria as well as the relative abundance of specific forms will change rapidly soon after the samples of water and mud are exposed to surface temperature, due either to a change in environmental conditions or to a change in food supply, it is quite essential that the collected samples should be subjected to immediate investigation. As a result of these considerations, the laboratory on the "Atlantis," during the cruise made in August, 1932, was so outfitted that it was possible to carry out the bacteriological studies within a few minutes or at most hours, after the samples of water, bottom mud, or sand were brought up from their respective depths.

Because of the extensive hydrographic and biological investigations which have been carried on in the Gulf of Maine, this region is particularly well adapted to a study of the relative abundance and activities of marine bacteria. Such a study was further aided by the fact that other biological and chemical investigations were carried on simultaneously on this cruise. The locations of the various stations at which samples were taken during this cruise are described in the first paper of this series.[2] Of the eight stations studied, five were in the Gulf of Maine proper and the remaining three in the shallow waters over Georges

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[1] Contribution No. 10 of the Woods Hole Oceanographic Institution and Journal Series Paper of the New Jersey Agricultural Experiment Station.

[2] Rakestraw, Norris W., 1933. Chemistry of the Waters of the Gulf of Maine in August, 1932. *Biol. Bull.*, **64**: 149.

Bank. A detailed description of the methods used in these investigations will be presented later, when the bacteriological results of this expedition will also be compared with those obtained from waters and sea bottoms near shore. Only a brief summary of the investigations carried out on and in connection with this cruise of the "Atlantis" will be reported here. An attempt will be made to interpret the results of these investigations in the light of the chemical conditions of the waters and nature of the marine bottom, as well as to correlate the results of the bacteriological investigations with the results of the chemical and planktonological studies made by the other investigators on this cruise.

Abundance of Bacteria in Sea Water and in the Sea Bottom

The relative numbers of bacteria in the sea are usually determined by means of the plate method. This method has numerous limitations and can at best give only a very approximate idea of the actual abundance of the bacteria, both in the water and in the sea bottom. Although new methods have been introduced recently, consisting in direct staining of the bacteria in the water, after they have been concentrated by filtration or centrifuging, these methods are still in an experimental stage and were not used on this expedition. If the various limitations of the plate method are kept in mind and if broad generalizations are avoided, the results obtained even by this procedure can be made to throw considerable light upon the relative abundance and nature of the bacterial population of the sea.

The numbers of bacteria were determined in the water from the different stations at three different depths, namely, the surface, 50 meters, and the bottom. At one station, water from two additional depths of 30 and 100 meters was also investigated. The surface samples were taken with ordinary sterilized and evacuated glass tubes provided with a sealed glass tip which was broken about one foot below the surface of the water. Deeper samples were taken in heavier-walled glass tubes having a specially constricted neck to prevent the rubber stopper being forced into the tube by the pressure of the water. The inlet tube in this case was made of a capillary glass tube having a 1 mm. bore. Tubes of this type were used successfully to a depth of 330 meters but the lower limit of their usefulness has not yet been determined. Mud samples were taken by means of a sampler consisting essentially of a brass tube carrying a weight sufficient to force it some distance into the mud when the bottom is reached. Into the brass tube is fitted a glass tube having a check valve attached to its upper end. This allows water to stream through the tube during its descent. While raising the sampler the valve closes and prevents any downward pressure upon the mud core within. By sterilizing the glass tube before use it is possible to eliminate any bacterial contamination of the mud sample other than the contamination of the walls of the glass tube by the water passing through it while descending. This may be easily eliminated by taking the sample to be studied from the center of the core and discarding the surface which has been in contact with the glass tube.

The water was plated undiluted and diluted 10 times, while the mud was plated in dilutions of 1 : 10, 1 : 100 and 1 : 1000. Previous experiments, which will be reported elsewhere, have shown that the following medium is satisfactory for the development of marine bacteria, capable of growing on the ordinary plate:

Agar	15 grams
Glucose	1 gram
Peptone	1 gram
K_2HPO_4	0.05 gram
Sea water	1000 cc.

Table I

Abundance of bacteria in sea water. Total numbers in 1 cc. of water.

Station Number		1329	1330	1331	1332	1333	1334	1335	1336
Total depth of water, *meters*		256	207	230	346	230	71	74	64
Depth of sample, *meters*	Surface	10	3	4	2	380	16	92	6
	30			0					
	50	1	1	2	1	2			
	100			1					
	Bottom	6	0	1	1	3	25	41	27

The plates were incubated at air temperature and the colonies counted at the end of 7 and 10 days. All the samples of water and mud were plated out within a few minutes after being taken.

The total numbers of bacteria in the water, as determined by the plate method, are given in Table I. With respect to their bacterial content, the stations may be divided into two groups, the first containing the five deep water stations and the second the three shallow stations. With the exception of the surface water of Station 1333, the bacteria in the water of the first group, namely, Stations 1329 to 1333 inclusive, were uniformly so extremely low in number, that it is difficult to draw reliable conclusions in regard to their distribution. It would seem, however, that the bacteria were slightly more numerous at the surface than in the lower depths of water. There was no appreciable variation between the different other depths sampled, either at a single station or between different stations. Neither was there any appreciable variation in the bacterial content of the surface water of the different stations of this group. The very high content of the surface water of Station 1333 was probably due to the sample inadvertently being taken near floating seaweed or the introduction of some other contamination. The water samples from the lower levels of this station resembled those from the other deep water stations in their bacterial content.

The bacteria in the water of the second group of stations were uniformly much more numerous than in the water from the deeper stations. In view of the other results to be reported below, this may be ascribed to the higher phytoplankton content of this water. In two of the three stations the bottom water samples were considerably higher in bacteria than the surface samples. One must keep in mind the fact that the bottom of the last group of stations was sandy, while the bottom of the first four stations was mud. To what extent the deposits at the bottom of the ocean directly affect the bacterial content of the water for any very great distance above, except in turbulent waters, still remains to be determined. Because of the stray of the sampling cable from the perpendicular and the rise and fall of the ship with the waves, it is not possible to secure water samples with the apparatus used nearer than 2 or 3 meters from the bottom. The results, therefore, cannot be taken as indicating the bacterial content of the layer of water in contact with the mud bottom. It is possible to secure water samples nearer the bottom at shallow stations, as in the case of the second group. This, together with the fact that the water at these stations is rather turbulent, may indicate the transfer of sufficient nutrient material from the bottom deposit to the water immediately above to support the greater number of bacteria found in these waters.

The more uniform distribution of the bacteria in the water at the shallower stations is also correlated with the even distribution of the diatoms. This is probably due to the stronger vertical mixing of the waters, as shown by the even distribution of temperature and salinity.

The results reported above agree with those of various other investigators in indicating that the numbers of bacteria in sea water are relatively low and quite uniform in distribution. In an effort to secure further information concerning the presence of bacteria in sea water, the association of these organisms with the plankton forms was investigated. By towing with plankton nets samples of both zoöplankton and phytoplankton were collected. A number 20 silk net was used, which left out the nannoplankton. The zoöplankton consisted largely of copepods, while the phytoplankton was made up almost entirely of diatoms with a few *Ceratium* cells present. This material was collected in the glass jar at the bottom of the net and portions of it immediately plated out. The numbers of bacteria in the various tow waters are given in Table II.

The water containing the diatom plankton is extremely rich in bacteria. It was not possible to make determinations of the actual quantity of plankton present in the samples. Apparently the zoöplankton has fewer bacteria associated with it than the phytoplankton. Dr. Gran estimated that the sample obtained from Station 1336 contained 11,900 planktonic organisms per liter of tow water, of which 11,100 were diatoms. It is of considerable interest to compare the relative numbers of diatoms in the tow and in the sea water at this station with the numbers of bacteria in the tow water and in the sea water. In the case of the zoöplankton (Station 1331), there were 225 times as many bacteria in 1 cc. of the tow as in 1 cc. of the corresponding water, thus giving a ratio of the bacteria in the tow to the bacteria in the water of 225 : 1.

Table II

Abundance of bacteria in tow water. Numbers in 1 cc. of tow.

Station No.	Type of plankton	Total bacteria	Agar-decomposing bacteria	Percentage agar-decomposing bacteria
1331	Zoöplankton	890	0	0
1335	Diatom plankton	36,700	2,100	5.7
1336	Diatom plankton	37,500	2,500	6.7

In the case of the diatom plankton, the ratio between the bacteria in the tow and the bacteria in the water (taking the average of the surface and bottom samples) was 500 : 1 and 2,270 : 1, for Stations 1335 and 1336 respectively. It is important to note that Dr. Gran found that the ratio for the respective numbers of diatoms in the tow and in the water was about 1,667 : 1. This indicates that a definite parallelism exists between the bacteria and the diatoms in the water and in the plankton. The actual existence of such relations is substantiated by the fact that the bacteria from the diatom tow showed a considerable abundance of agar-liquefying organisms. While one encounters only infrequently such organisms in the free water, usually only very near shore, and in the mud, they were found to make up 5.7 and 6.7 per cent of the total bacterial flora in the plankton tow, as determined by the agar plate method. It is interesting to note that the bacteria found in the copepod-plankton did not include any agar-liquefying forms.

It is quite possible, therefore, that bacteria exist only to a very limited extent in the free water of the sea, but are largely attached to the plankton organisms, living upon the dead members of the plankton or upon the excretion products of the cells, upon the cell membranes, especially the mucilaginous substances secreted by certain algæ, etc. One need not, of course, imagine that if such an associative growth exists, the numbers of higher plankton forms and bacteria will always be parallel. One can readily imagine that the bacterial maximum may be attained after the diatom and copepod maximum. This has been brought out in an experiment carried out in cooperation with Dr. Gran and described by him elsewhere, in which the numbers of diatoms and of bacteria were determined simultaneously in artificial cultures of diatoms in sea water (Table III). The rise in numbers of both groups of organisms appears to have been directly related. The increase in bacterial numbers was much greater proportionately than that of the diatoms. When the multiplication of the latter reached a maximum, the numbers of bacteria continued to rise further, while the numbers of diatoms began to fall. This may be noted at the 7-day period when the diatoms had dropped to their original level, while the bacterial numbers were still increasing. This is to be expected since the death of the diatoms provides a source of food for the saprophytic bacteria. The numbers of bacteria dropped slowly and, within the course of the experiment, did not fall to the level of the original water as did the diatoms, indicating a greater resistance of bacteria to the artificial culture conditions.

TABLE III

Comparative numbers of diatoms and bacteria in diatom cultures. Numbers in 1 cc. of culture water.

	Original water	After 3 days growth	After 7 days growth	After 12 days growth
Diatoms........	177	1,026	178	165
Bacteria........	346	20,600	22,800	13,900

Because of the large numbers of bacteria capable of decomposing agar found associated with the diatoms of Stations 1335 and 1336, it was of interest to follow their behavior in this experiment. In the original water the number of agar-decomposing bacteria was of the order of 2 or 3 cells per cubic centimeter. At the end of the 3-day culture period this figure had risen to 50 cells per cubic centimeter. At none of the later periods did any agar-decomposing bacteria appear on

TABLE IV

Abundance of bacteria in the sea bottom. Numbers in 1 gram of mud or sand, dry basis.

Station Number	1329	1330	1331	1332	1335	1336
Surface	5,400	4,700	830	3,000	1,280	670
10 cm	1,720	1,700	140	290		

the plates. The disappearance of these bacteria may be ascribed to their overgrowth by other forms better adapted to the cultural conditions. This is shown by the fact that while the colonies on the plates from the original water indicated the presence of a considerable variety of bacterial forms, in the last plating from the cultures only one or two types remained.

The terrigenous bottom deposits of the Gulf of Maine contain varying amounts of organic matter originating partly from the organisms living in the sea bottom and sinking from the water above, but probably more largely from the organic matter present in a partly decomposed state in the sediments brought in from land. These forms of organic matter furnish the energy for the growth of the bacteria in the marine bottom. The total organic matter content in the mud and sand bottoms of the stations investigated is reported later, while a chemical study of the organic matter in sea bottoms or marine humus is reserved for another publication.

TABLE V

Presence of Anærobic Bacteria in the Marine Bottom

Medium		Solution				Agar			
Dilution		10		100		10		100	
Station No.	Depth of mud	1	2	1	2	1	2	1	2
1329	0–30 cm.	+	+	+	0	5*	2*	0*	2*
	30–60 cm.	+	0	+	0	12	2	4	8
	60–90 cm.	+	0	0	0	+	+	0	0
1330	0–30 cm.	+	+	+	0	30	18	10	8
1331	0–30 cm.	0	0	0	0	?	?	0	0

+ Indicates growth and gas formation.
* Figures = numbers of colonies in the agar shake-tube.

Mud samples for bacteriological investigation were obtained from Stations 1329 to 1332. The bottom at Station 1333 was made up of pebbles and no sample could be secured. The sand bottom of Stations 1334 to 1336 could not be sampled by the procedure outlined above because of the sand slipping from the tube as it was withdrawn from the bottom. However, at two of these stations sand samples were obtained by means of a Petersen grab. The numbers of bacteria found in these samples, both at the surface of the sample and at a depth of 10 cm., in the case of the mud bottoms, are given in Table IV.

TABLE VI

*Numbers of bacteria in sea water and in mud, as shown by the dilution method, using gelatin as the medium for bacterial development.**

Station	Depth †	Dilution	Water	Mud
		cc.		
1329	Surface	0.1		++
1329	Surface	0.01		++
1329	Surface	0.001		++
1329	10	0.1		++
1329	10	0.001		+
1330	Surface	0.01		++
1330	Surface	0.001		+
1330	Surface	0.0001		0
1330	10	0.01		++
1330	10	0.001		0
1331	Surface	0.01		++
1331	Surface	0.001		++
1331	Surface	0.1	+	
1331	Surface	0.01	0	
1331	30	0.1	0	
1331	30	0.01	0	
1331	50	0.1	+	
1331	50	0.01	0	
1331	100	0.1	+	
1331	100	0.01	+	
1331	215	0.1	0	

* + indicates growth after 3 weeks incubation; ++ indicates very good growth.

† Depth in water, is, in meters, from surface of water; depth in mud is, in centimeters, from surface of mud.

The bacterial numbers in the surface layer of the mud were found to vary from 830 to 5400 per gram of dry mud. These figures for the mud may be compared with the values of 670 and 1280 per gram of dry sand at Stations 1335 and 1336. The two samples of mud (1329 and 1330) which contain more organic matter contain also more bacteria; variations which occur in the different mud samples may be ascribed to differences in organic matter content or to physical variations.

The total number of organisms decreases rapidly with the depth of mud. Thus at Station 1329, at a depth of 10 cm., the number of bacteria is only 1,720 as compared to 5,400 at the surface. The decrease is even more noticeable in the mud of Station 1332. The bacteria do not disappear rapidly, however, below the surface layer of the mud, but extend to considerable depths, as shown by the data for the presence of anærobic organisms (Table V). These bacteria were determined by the dilution method, using liquid medium kept under anærobic conditions, and by the shake-tube method, using agar medium. The results indicate the widespread occurrence of anærobic bacteria near the surface of the mud and their presence to depths as great as 90 cm. below the surface.

There is no doubt that the numbers of bacteria found in sea water and in the sea bottom by the plate method represent only a fraction of the total numbers of bacteria. This is due to the limitations of the method, whereby only a part of the bacterial population develops on the plate into colonies. In order to check this, several samples of water and mud were diluted and 1 cc. portions of the various dilutions added to sterile portions of 10 per cent gelatin in sea water. The results presented in Table VI show that whereas the plate method gave only 1 to 4 cells in 1 cc. of water from Station 1331, the dilution method with gelatin as a medium gave positive growth in some instances with 0.1 cc. of water and in some even with 0.01 cc. of water, indicating that at least 10 to 100 bacterial cells were present in 1 cc. of the water. In the case of the mud samples, abundant positive growth was obtained in most instances with 0.001 gram.

Results of bacterial investigation of the water and bottom samples of the Barents sea, recently reported by Butkewitch,[3] also bring out the fact that the dilution method may give 10 to 100 times as many bacteria as the plate method. The direct microscopic method gives even larger numbers, usually a thousand times greater than those obtained by the plate method. This difference is due to the fact that not only living but also dead bacteria are counted by the microscope, while many bacteria unable to develop on artificial media are seen with the microscope.

Occurrence of Specific Groups of Bacteria in the Sea

A number of bacteria active in certain specific processes, which are no doubt of considerable importance in the metabolism of the sea, were demonstrated both in the sea water and in the sea bottom. It is sufficient to mention here the presence of ærobic and anærobic nitrogen-fixing bacteria, of nitrifying bacteria, of agar-liquefying and cellulose-decomposing bacteria, of nitrate-reducing bacteria, of various spore-forming and non-spore-forming bacteria, and of a number of heterotrophic ærobic as well as anærobic bacteria. Some of these organisms will be discussed in detail later, while others need only be mentioned here in passing.

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[3] Butkewitch, W. C. Method of Bacteriological Investigation and Certain Data on the Distribution of Bacteria in the Water and Bottom of Barents Sea. Trans. Russian Oceanogr. Inst., 2: No. 2, 1932.

At least three types of cellulose-decomposing bacteria were demonstrated to be present in the sea. They belong largely to the groups described by Winogradsky as *Cytophaga, Cellvibrio,* and *Cellfalcicula.* They produce yellow and orange pigments or no pigment at all. They were found in the water from Station 1329 and to a less extent in the mud of the same station, but they were especially abundant in the tow water collected at Station 1336. This phenomenon may suggest the probability that these organisms, as well as the agar-liquefying bacteria, also present abundantly in the diatom tow, as pointed out previously, are not found to any great extent in the free water or in the mud, but develop in close connection with the growth of the plankton organisms, especially the phytoplankton; one would expect that the latter would offer a logical substrate for the growth of such bacteria. Some of these bacteria were able to attack both cellulose and agar, while others attacked cellulose alone and still others grew upon agar alone.

The agar-liquefying bacteria are represented in the sea by a number of different groups. They produce yellow, pink, or brownish pigments or no pigment at all. Most of them are ærobic, while some are anærobic. A detailed description of these organisms will be published in a later study. Chitin-decomposing bacteria have also been demonstrated in the sea water but these organisms have as yet been insufficiently studied.

It is commonly assumed that the sea harbors very few spore-forming bacteria. This may be true of the water itself; the sea bottom, however, especially the mud bottom, was found to harbor large numbers of such bacteria. The spore-forming bacteria could best be demonstrated in the nitrogen-free media inoculated with various dilutions of the mud samples. Many of the spores were found to have the typical appearance of *Clostridium,* so that one came to expect the development of these organisms in the nitrogen-free media inoculated with mud. This organism was accompanied by other bacteria, some of which were spore-forming. Many spore-forming bacteria were found abundantly in the calcium acetate-sodium nitrate medium used for the study of nitrate-reducing bacteria.

Anærobic bacteria were demonstrated by the use of liquid and solid media sealed with vaseline. Gas formation in the liquid media and colony development in the shake-tubes were taken as evidence of the development of these organisms. The ordinary peptone-glucose medium was used for this purpose. The cultures were inoculated with material from the mud profiles (Table V). The formation of colonies in the anærobic cultures brought out the fact that at least several hundred anærobic cells are present in each gram of moist mud, even to a depth of 60 cm. of mud.

Occurrence of Nitrogen-fixing Bacteria in the Gulf of Maine

The problem of nitrogen-fixation in the sea has always aroused considerable interest due to the importance of this phenomenon in marine metabolism. If it is true, as some oceanographers assume, that the sea produces as much plant and animal life as a similar area of land, the amount of nitrogen required for the synthesis of these numerous forms of life is quite enormous, since the nitrogen content of the various representatives of marine life varies from 0.7 per cent in the case of certain algæ, such as *Fucus*, to 10 per cent in the case of various animals which range from the lowly copepods to the large fish and mammals.

By the use of silica-gel plates and liquid media containing a layer of sand, the presence in the sea of the two important groups of non-symbiotic nitrogen-fixing bacteria, namely the aerobic *Azotobacter* and the anærobic *Clostridium*, was definitely demonstrated. The occurrence of organisms in the sea water and sea bottom of the Gulf of Maine, capable of growing on nitrogen-free liquid media, is brought out in Table VII. The nitrogen-fixing organisms were found both in the surface waters and in the marine bottom. Samples of water taken from Station 1331 at depths of 30, 100, and 215 meters gave largely negative results.

A detailed description of the methods used in this study as well as the nature of the organisms found and their nitrogen-fixing capacity will be reported in another publication. It suffices here to say that a nitrogen-free medium containing a carbon source (glucose, mannitol, salts of organic acids), a source of phosphate and organic iron was used. In the case of the plates, this medium was added to silica-gel prepared in large Petri dishes (15 cm. in diameter); it was then subjected to dialysis in tap water, and the plates finally soaked for a few minutes in boiled sea water. Each plate contained 2 grams of carbon source. The liquid medium was placed in large test tubes with a layer of sand on the bottom and sterilized.

The silica gel plate inoculated with mud from Station 1329 gave a

TABLE VII

Distribution of bacteria capable of growing on nitrogen-free media in ocean waters and marine bottoms

Station No.	Depth†	Dilution in cc. or grams	Sea Water						Marine Bottom					
			Mannitol			Glucose			Mannitol			Glucose		
			Growth*	Gas	Organisms‡	Growth	Gas	Organisms‡	Growth	Gas	Organisms‡	Growth	Gas	Organisms‡
1329	Surface	1.0	T, P	0	Bac.	P	0	Rods	—	—	—	—	—	—
1329	Surface	0.1	T	0	Bac.	T, P	0	—	T, P	+	Bac.	T	+	Bac.
1329	Surface	0.01	0	0	0	0	0	0	P	+	Bac.	T	—	—
1330	Surface	1.0	—	—	—	—	—	—	T, P	+	l.r.	—	+	sm.r.
1330	Surface	0.1	—	—	—	—	—	—	T	+	—	P, T	+	—
1331	Surface	1.0	T, P	—	sm.r.	T	0	sm.r.	—	—	—	—	—	—
1331	Surface	0.1	—	—	—				T	+	Bac.	T	+	Bac.
1331	Surface	0.01	—	—	—				T	+	Bac.	T	+	Bac.
1331	30	1.0	—	—	—	—	—	—						
1331	50	1.0	T, P	0	0	T	0	—						
1331	100	1.0	P	0	0	T	0	—						
1331	215	1.0	0	0	0	T	0	—						
1332	Surface	0.1	—	—	—	—	—	—	P	+	Bac.	T	+	l.r.
1332	10	0.1	—	—	—	—	—	—	—	—	—	T	+	—
1333	Surface	0.1	T, P	0	—	T, P	0	—	—	—	—			
1333	Deep	0.1	—	—	—	T, P	0	sm.r.						
1334	Surface	0.1	T	0	—	T	0	—	—	—	—			
1334	Deep water	0.1	T, P	0	sm.r.	—	—	—	—	—	—			
1335	Surface	1.0							T, P	+	sm.r.	T, P	+	l.r.
1336	Surface	1.0							P	—	sm.r.	P	+	sm.r.

* P = pellicle formation, T = turbidity.

† Depth in water, in meters, is from surface of water; depth in mud, in centimeters, is taken from surface of mud.

‡ Bac. = spore-forming rods; sm.r. = small rods; l.r. = large rods.

typical development of *Azotobacter chroococcum*. This organism was readily isolated and grown in pure culture. On nitrogen-free sea water media, with glucose or mannitol as a source of energy, it fixed four to six milligrams of nitrogen for one gram of carbohydrate in 21 days.

Occurrence in the Gulf of Maine of Bacteria Capable of Oxidizing Ammonium Salts to Nitrites

If there is any one group of bacteria that has attracted the greatest attention of oceanographers and of bacteriologists alike, it is the group of nitrifying bacteria, or those organisms which produce nitrites from ammonium salts and nitrates from nitrites. A review of the various theories proposed to explain the occurrence of nitrite and nitrate in the ocean would in itself require considerable space. It suffices here to bring out only the results of the "Atlantis" expedition, to enable one to draw some very definite conclusions. It is important to emphasize in this connection that negative results do not necessarily mean the absence of the specific organisms but merely that the possibility exists that the medium used for demonstrating these organisms and the conditions essential for their development were not particularly favorable. Positive results are, however, much more important.

Of the two processes leading to the formation of nitrate, the first one, namely the oxidation of ammonium salts to nitrite, is usually considered to be more specific and more important, hence this process was more emphasized during this cruise.

Preliminary investigations of the water taken near shore brought out the fact that nitrite formation can take place in a sea water medium if proper conditions are provided. Three methods were used for this purpose: (1) Sand and nutrient salts—$CaCO_3$, $(NH_4)_2SO_4$, K_2HPO_4—were added, in proper concentration to flasks and these were sterilized; the fresh sea water taken from the various stations and at the different depths was introduced directly into these flasks. (2) In another set of flasks sand taken from Georges Bank was used in a fresh state and water taken from some of the stations was added. (3) In a third experiment, sea water, sand, and chemicals were placed in flasks, sterilized, and inoculated with mud from the various stations. The results obtained on the "Atlantis" cruise are presented in Table VIII. These results prove definitely that nitrite-forming bacteria are completely absent in the free sea water or are present there only in very limited numbers, while the sea bottom contains an abundance of such organisms. In the case of the mud, the presence of nitrifying bacteria was demonstrated at quite appreciable depths. This tends to demonstrate that nitrite formation and probably also nitrate formation take place in the sea bottom. The nitrite and nitrate ions will then diffuse into the water and will be gradually brought upwards by ocean currents, etc. Near the surface of the water, where active photosynthesis takes place, the nitrates tend to disappear due to the fact that they are rapidly consumed by the phytoplankton organisms.

Occurrence in the Sea of Bacteria Capable of Reducing Nitrates to Nitrites and to Atmospheric Nitrogen

The presence in the sea of bacteria capable of reducing nitrates has attracted considerable attention from oceanographers, largely as a result of the hypothesis proposed by Brandt,[4] who attempted to base the whole system of marine metabolism on the occurrence and activities of these organisms. Preliminary studies in this laboratory have shown that when a liquid medium containing Ca-acetate, $NaNO_3$, and a layer of sand is inoculated with shore water or with mud, active nitrite formation will take place, accompanied by abundant gas evolution. When this medium was inoculated with mud, even in a dilution of 1:1000, the nitrate disappeared completely within 5 days. In other words, both the water and the mud close to the shore contain an abundance of organisms

Table VIII

Presence of nitrifying bacteria in sea water and sea bottom

Nitrite-forming bacteria in water			Nitrite bacteria in sea bottom		
Station No.	Depth of water	Nitrite formation in 20 days	Station No.	Depth of mud	Nitrite in 11 days
	meters			*cm.*	
1329	Surface	−	1329	0–30	+++
1329	Bottom	−	1329	30–60	++
1330	Bottom	−	1329	60–90	trace
1331	Surface	−	1330	0–30	++
1331	30	trace	1330	30–60	trace
1331	50	+	1330	60–90	trace
1331	100	−	1331	0–35	trace
1331	215	+	1336	Sand	+
1331	Bottom	−			
1332	Surface	+			
1332	50	+			
1332	100	+			
1333	Surface	trace			
1334	Surface	−			
1334	Bottom	trace			

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[4] Brandt, K. Ueber den Stoffwechsel im Meere. Wiss. Meeresunters. Kiel, N. F., **4**: 213–230, 1899; **6**: 23–79, 1902; **18**: 185–429, 1916–1920; **19**: 251–253, 1919.

capable of reducing nitrates to nitrites, as well as of reducing the latter completely to atmospheric nitrogen.

For the investigations carried out on the "Atlantis" cruise, a medium similar to the above and containing 10 grams Ca-acetate, 1 gram $NaNO_3$, 0.5 gram K_2HPO_4, and a trace of iron per liter of sea water was placed, in 25 cc. portions, in large test tubes containing a layer of sand. The medium was sterilized at 15 lbs. for 15 minutes. The tubes were inoculated immediately after the samples were taken, using various dilutions of water and mud. After two weeks incubation at air temperature, the cultures were examined microscopically and tested for nitrite (Tables IX, X).

Table IX

Relative abundance of nitrate-reducing bacteria in sea water

Station number	Depth of water	Dilution of water	Growth of bacteria	Microscopic examination	Gas evolution	Nitrite formation
	meters	*cc.*				
1329	Surface	1.0	Turbid	Large round cells and curved rods	–	+++++
1329	Surface	0.1	–	–	–	–
1329	Surface	0.01	–	–	–	–
1331	Surface	1.0	Turbid	Minute coccobacilli	–	+++++
1331	Surface	0.1	Slight turbidity	Minute coccobacilli	–	+++++
1331	Surface	0.01	–	–	–	–
1331	30	1.0	Turbid	Cocci and bacilli	–	+++++
1331	30	0.1	Turbid	Cocci and bacilli	–	+++++
1331	30	0.01	–	–	–	–
1331	50	1.0	Turbid	Cocci and bacilli	–	+++++
1331	50	0.1	–	–	–	–
1331	100	1.0	–	–	–	–
1331	100	0.1	–	–	–	–
1331	215	1.0	Slight turbidity	Long rods and cocci	–	+++++
1331	215	0.1	–	–	–	–
1333	Surface	0.1	Turbid	Small coccobacilli	–	+++++
1333	Surface	0.01	Turbid	Small coccobacilli	–	+++++
1333	Surface	0.001	Turbid	Small coccobacilli	–	+++++
1333	Bottom water	0.1	–	–	–	–
1333	Bottom water	0.01	–	–	–	–
1334	Surface water	0.1	–	–	–	–
1334	Surface water	0.01	–	–	–	–
1334	Deep water	0.1	–	–	–	+++++
1334	Deep water	0.01	–	–	–	–

Of all the cultures inoculated with water or with mud, only one gave positive gas evolution, namely, the one inoculated with 0.1 gram of mud from Station 1329. Positive growth of bacteria was always accompanied by a reduction of the nitrate to nitrite. This experiment tends to prove that the waters and the mud in the Gulf of Maine are able to reduce nitrates only to nitrites, but not to atmospheric nitrogen. In the case of the waters, nitrate reduction was active to a depth of 50 meters. Morphologically, the organisms developing in these cultures were found to differ considerably with the different inocula. Some were plain rods, others resembled *Azotobacter* cells, while still others were undeniably spores.

Table X

Relative abundance of nitrate-reducing bacteria in the mud

Station number	Dilution of mud	Growth of bacteria	Microscopic examination	Gas evolution	Nitrite formation
1329	0.1	Turbid	Spores	++	++
1329	0.01	Turbid	Few cells	–	+++++
1329	0.001	0	0	0	0
1330	0.1	Turbid	Oval rods, spores (?)	–	+++
1330	0.01	Turbid	Oval rods, spores (?)	–	++++
1330	0.001	Turbid	Oval rods, spores (?)	–	–
1330	0.0001	–	–	–	–
1331	0.1	Turbid	Spores	–	+++++
1331	0.01	Some turbidity	Spores	–	+++
1331	0.001	Some turbidity	–	–	+++
1331	0.0001	–	–	–	–
1332	0.1	Turbid	Spores, rods	–	+++++
1332	0.01	Turbid	Spores, rods	–	+++++
1332	0.001	–	–	–	–

Explanation of Nitrate Cycle in the Sea on the Basis of the Occurrence of Ammonia-oxidizing and Nitrate-reducing Bacteria

The results of the investigations carried out in connection with this expedition prove beyond any doubt that the marine bottom, both sand and mud, contains bacteria capable of oxidizing ammonia to nitrite, and later to nitrate; the process of nitrate formation in the sea must, therefore, be considered as bacteriological in nature. The results also show that the sea water and marine bottom contain bacteria capable of reducing nitrate to nitrite, in the presence of sufficient energy material, in the form of readily available carbon compounds. The fact that marine humus cannot be used as such a source of energy, while the amount of available carbohydrate in the mud is very limited, tends to emphasize that this process in the sea is only of questionable importance.

The problem finally remains to establish whether the limited occurrence of nitrite in sea water is due to the oxidation of the ammonia by nitrifying bacteria or to the reduction of nitrate by other bacteria. There is no doubt that the oxidation takes place chiefly at the bottom of the ocean; further that while the formation of nitrite from ammonium salts, under proper conditions of culture and with bottom material as an inoculum, can be readily demonstrated, nitrate formation takes place, under laboratory conditions at least, at a much later date. Reduction of the nitrate can be expected to take place both in the sea water and in the sea bottom; however, this process requires a supply of organic matter as an available source of energy. This can only be obtained through the photosynthetic activities of the planktonic organisms. It is no mere coincidence, therefore, that the maximum nitrite formation corresponds well with the maximum oxygen content in the water or the maximum photosynthetic activities.

The following hypothesis suggests itself at this particular point: Decomposition of the organic nitrogenous compounds takes place in the sea water but largely on the sea bottom, with the result that the ammonia is then liberated. This ammonia is rapidly oxidized by specific bacteria living in the bottom to nitrite and later to nitrate. This nitrate remains in the sea bottom and is not reduced, due to a lack of available energy for the nitrate-reducing bacteria and not to a lack of such bacteria. The small amounts of ammonia found in the sea water originate from the decomposition of the plant and animal residues in the plankton and in the water. The nitrate formed in the bottom gradually diffuses into the water, where it remains as such. On reaching the zone of photosynthetic activities, this nitrate is either consumed by the phytoplankton or is reduced by the nitrate-reducing bacteria to nitrite, which may also be gradually consumed by the plants. Very little nitrate reduction to gaseous nitrogen or complete denitrification is possible under normal sea conditions. Reduction of nitrate to nitrite does not mean necessarily any loss of nitrogen from the cycle of life in the sea.

ABUNDANCE OF MARINE HUMUS IN THE BOTTOM OF THE GULF OF MAINE

A series of mud samples was taken with the small glass tube from the first four stations and from one sand station, in order to determine the abundance as well as the variability in distribution of the organic matter in the sea bottom. The methods of analysis, as well as the importance of these results in determining the origin and nature of the organic matter or the marine humus in the sea will be discussed in a later publication. The organic matter is reported in terms of carbon and nitrogen. In order to calculate the organic matter content of the mud or sand from the carbon data, the factor 1.724, which has found extensive application in soil investigations, may be used, for reasons discussed elsewhere.

TABLE XI

Distribution of marine humus in the sea bottom, as shown by the carbon and nitrogen content. Percentage of dry material.

Station No.	Sample No.	Carbon content	Nitrogen content	C/N
1329	1	2.46	0.284	8.7
1329	2	2.50	0.290	8.6
1329	3	2.48	0.290	8.5
1329	4	2.46	0.282	8.7
1329	5	2.37	0.278	8.6
1329	Average	2.45	0.285	8.6
1330	1	2.65	0.304	8.7
1330	2	2.66	0.284	9.4
1330	3	2.62	0.306	8.6
1330	4	2.70	0.294	9.2
1330	5	2.81	0.288	9.7
1330	Average	2.69	0.295	9.1
1331	1	1.58	0.156	10.1
1331	2	1.54	0.148	10.4
1331	3	1.55	0.144	10.8
1331	4	1.36	0.133	10.2
1331	5	1.29	0.133	9.7
1331	Average	1.46	0.143	10.2
1332	1	0.82	0.072	11.4
1332	2	1.20	0.096	12.5
1332	Average	1.01	0.084	12.0
1335	1	0.084	0.008	10.5
1336	1	0.12	0.009	13.3

The results reported in Table XI show that while the variation in the organic matter content is very small in some of the stations, it is quite appreciable in others. These results point definitely to the conclusion that determinations based upon a single sample may frequently give quite inaccurate results.

The distribution of marine humus in the mud profiles taken from the various stations is given in Table XII. A heavy brass sounding tube was used for this purpose. The depth of the mud brought up by means of this corer ranged from 75 to 115 cm. As soon as brought up, the cores were divided into three or four sections and later each analyzed separately. It is important to remember, of course, that the mud was compressed by the sampler and that the actual depth of the mud was greater than that shown by the figures. The analyses of the total carbon and nitrogen were made on the materials which had been dried in an oven at 100° C. There has been some question, however, as to the influence of drying upon the changes in the nitrogen content of the mud.

Table XII

Relative distribution of marine humus with the depth of the mud profile. On basis of dry material.

Station No.	Depth of mud	Carbon content	Nitrogen content	C/N
	cm.	*per cent*	*per cent*	
1329	0–30	2.58	0.290	8.7
1329	30–60	2.45	0.250	9.8
1329	60–90	2.32	0.238	9.75
1330	0–30	2.74	0.296	9.25
1330	30–60	2.75	0.254	10.8
1330	60–90	2.60	0.236	11.0
1331	0–35	1.58	0.140	11.3
1331	35–65	1.64	0.130	12.6
1331	65–90	1.61	0.128	12.6
1331	90–115	1.57	0.126	12.4
1331	90–115 (reddish mud)	1.04	0.080	13.0
1332	0–25	0.67	0.050	13.4
1332	25–50	1.12	0.092	12.2
1332	50–75	1.43	0.096	14.9

The samples obtained from Station 1329 were, therefore, analyzed for total nitrogen both in a moist and in a dry condition and both sets of results calculated on a dry basis (Table XIII). These results show that while in some instances there is no appreciable difference in the nitrogen content of the mud as a result of its preliminary drying in the oven, in other cases the difference obtained may be appreciable. All the analyses reported here were made on the oven-dried (60–90° C.) mud, due primarily to the ease of handling and to the greater uniformity of the samples.

Stations 1329, 1330, and 1331 show a gradual even if only a very limited decrease of the humus content with an increase in depth of mud, especially on the basis of the nitrogen figures. This is accompanied by a gradual decrease in the nitrogen content of the humus. This is due either to the greater decomposition of the nitrogen complexes with the age of the humus or to a difference in the nature of the materials deposited at different times. One is struck, however, by the great relative uniformity of the organic matter in the mud throughout the whole profile. One exception to this is found in one of the samples from the 90–115 cm. layer of Station 1331. This section of the profile was distinctly different in color from the rest of the profile and even from the duplicate part of another profile, being much redder in color. This proves again that even at one and the same station the mud may not be very uniform; this was brought out in Table XI for the data obtained from this station.

In the case of Station 1332, there is an increase in the humus content with an increase in the depth of the profile, as shown by both the carbon and nitrogen figures. This is due entirely to a difference in the nature of the material deposited at different periods, the surface material being much coarser than the lower layers. The amount of humus in the sand bottoms is very limited, usually equivalent to about 0.1 per cent carbon on the basis of the dry material. In view of the fact that no apparatus has been devised as yet to enable one to take a deep core of the sand bottoms, it is impossible to state how this humus content changes with the depth of the bottom. No information is available concerning any differences that may exist in the chemical nature of the humus in the mud and sand bottoms.

TABLE XIII

Influence of drying of marine mud upon its nitrogen content. On basis of dry mud.

Depth	Moist mud analyzed	Dry mud analyzed
cm.	*per cent N*	*per cent N*
0–30	0.306	0.290
30–60	0.282	0.250
60–90	0.239	0.238

On comparing the data of the humus content in the mud (Table XI) with those of the bacterial numbers (Table IV), one finds a certain definite correlation. This is especially true when one compares Stations 1329 and 1330 with a high humus content, on the one hand, with Stations 1331 and 1332 with a low humus content, on the other. One must keep in mind, of course, the fact that the numbers of bacteria were determined either in the very surface layer of the mud or in the 10 cm. layer, while the organic matter content was determined in the total core which ranged in depth from 15 to 25 cm. The bacteria are present most abundantly in the uppermost surface layer, where the organic matter is of recent origin and no doubt different chemically from the organic matter in the lower depths of the mud. The surface layer of the mud can be looked upon as the layer most active bacteriologically. The same is probably true of the sand, where the relative abundance of bacteria is considerably greater than the relative humus content. The abundance of humus can, therefore, be looked upon as only one factor controlling bacterial numbers and activities.

SUMMARY

A bacteriological survey has been made of the waters and bottom sediments in the Gulf of Maine and Georges Bank. The samples of water, plankton tow, and sedimentary material were taken under sterile conditions and subjected immediately to bacteriological analysis as soon as brought on board the "Atlantis."

The agar-plate method was used for the enumeration of the numbers of bacteria. This was supplemented to a limited extent by the dilution method. Various specific media were used to determine the distribution and relative abundance of certain groups of bacteria which are believed to take part in important marine processes.

The results obtained demonstrated the fact that the bacterial population of the sea can be divided into three groups on the basis of their habitat: (1) those forms which live in the sea bottom, especially in the surface layers; (2) those bacteria which live in the free water, this being possible only when the water contains in solution organic and inorganic substances which can serve as nutrients for the bacteria; (3) those bacteria which live largely upon or in association with the plankton organisms.

Sea water is a rather poor medium for the growth of bacteria, while the marine bottom is comparatively richer in the total number of bacteria capable of developing on the plate and in solution media. Mud bottoms contain more living bacterial cells than sand bottoms. However, the waters above the sandy bottom were found to contain many more bacteria than the waters above the mud bottoms. This may be due to the greater abundance of plankton organisms, especially diatoms, in the shallower seas with the sandy bottom, to the greater mixing of the waters, or to the greater absorption of bacterial cells by the mud bottom material than by the sand bottom.

The numbers of bacteria obtained by the plate method represent only a part of the bacterial population of the sea. This was shown by the fact that the dilution method, using gelatin as a medium for bacterial development, gave higher numbers than the plate method.

A decided parallelism was observed between the abundance of diatoms in the sea and abundance of bacteria. A comparatively large number of these bacteria were agar-liquefying organisms. In the artificial culture of diatoms, the numbers of bacteria were found to increase with the development of the diatoms. However, when the latter reached a maximum and began to die out, bacterial numbers did not diminish very rapidly; these bacteria seemed to be largely limited to a few specific types, the importance of which in marine processes still remains to be determined. These results seem to point definitely to the fact that the development of phytoplankton in the sea is accompanied closely by bacterial development. The bacteria feed upon the excretion products of the diatoms, algæ, and animal forms and probably upon these plankton forms themselves as soon as they die, thus bringing about their rapid disintegration and liberation of the nutrient elements in an available form.

Anærobic bacteria were found abundantly in the marine mud; these bacteria were present in the mud even at considerable depths. Their presence points to continued decomposition of the plant and animal debris of the ocean on and in the ocean bottom, even with an insufficient supply of oxygen.

The bacterial population of the sea was found to consist of a number of types, some of which take part in well-known processes which are of great importance in the metabolism of the sea, such as nitrogen-fixing, nitrite- and nitrate-forming, nitrate-reducing, cellulose-decomposing, agar-decomposing, chitin-decomposing, and many others. The importance of many common bacteria, both ærobic and anærobic, mostly non-spore-forming but also spore-forming, in marine processes still remains to be determined. The presence of nitrogen-fixing bacteria, comprising both the ærobic *Azotobacter* and the anærobic *Clostridium,* has been definitely demonstrated. One strain of the first group was isolated in pure culture and was found to be *Azotobacter chroococcum,* which fixed considerable quantities of nitrogen when grown on artificial culture media, with various carbon sources.

Bacteria capable of oxidizing ammonium salts to nitrites were found in the sea bottom, but only seldom in the sea water. For demonstrating the presence of these organisms in the sea, a medium containing a layer of sand and $CaCO_3$ and covered with a shallow layer of liquid containing an ammonium salt and the necessary minerals was used.

Bacteria capable of reducing nitrate were found abundantly both in the water, especially at the surface layers and the zone of photosynthetic activities, and in the sea bottom. However, these bacteria were able to reduce nitrate only to nitrite and not to nitrogen gas; only in one instance, namely, in the mud from the first station taken on this expedition, was there present an organism capable of bringing about the last process.

The cycle of nitrate in the sea is explained as follows: The nitrogenous constituents of the plant and animal residues in the sea are decomposed with the liberation of ammonia, largely in the sea bottom. The ammonia is oxidized in the sea bottom to nitrite and later to nitrate. The latter gradually diffuses into the waters. It is not reduced in the sea bottom or lower layers of water, largely because of a lack of available energy material necessary for the activities of the nitrate-reducing bacteria. In the zone of maximum photosynthetic activity such energy is available, hence nitrate-reduction may take place, but only to nitrite, since bacteria capable of reducing it to nitrogen gas are lacking under those conditions. The nitrite thus formed may also be assimilated by the phytoplankton and does not represent any loss of nitrogen in the sea.

The marine humus (total organic matter) content of the mud bottom in the Gulf of Maine is found to be more or less uniform in composition. This humus is best calculated from the total organic carbon. It was found that determinations based upon one sample may not give absolute evidence, since different samples may give considerable variation. The humus content usually decreases with the depth of the mud. In some stations, however, an increase in humus content with depth was observed. There is also a widening of the carbon-nitrogen ratio of the humus with an increase in depth. This points to a greater decomposition of the humus, especially of the nitrogenous complexes, with an increase in depth. An increase in humus content with depth of mud found in some stations merely points to the fact that the rate of deposition of the humus may vary considerably with different periods.

A definite parallelism was observed between the numbers of bacteria and abundance of humus in the mud.

A detailed review of the literature bearing upon the results presented in this report, as well as a detailed description of the methods used and a correlation of these results with the results of bacteriological investigations of the sea near shore, is reserved for a future publication.

THE OVARIAN CYCLE IN THE VIVIPAROUS TELEOST XIPHOPHORUS HELLERI

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A number of teleostean families include viviparous genera. These vary in type of reproductive adaptation from ovoviviparity, where the ova are merely retained in the ovary until hatching, to true viviparity where elaborate nutritive organs are formed by both parent and offspring. The Pœciliidæ, to which *Xiphophorus* belongs, includes oviparous, ovoviviparous, and truly viviparous forms, many of which are commonly raised in aquaria.

The first description of the development of a pœcilid is by Duvernoy (1844) on *Pœcilia surinamensis.* Wyman (1856) described the development of the "four-eyed fish," *Anableps gronovii,* which he found to be truly viviparous, the yolk-sac serving as an absorptive organ. Ryder (1882) described the development of *Gambusia* and its ovarian gestation. He again published (1886) his material as part of a longer paper on the development of viviparous osseous fishes in which he reviews all the early work. To this day his paper is the best single work treating with this phase of the subject. He found *Gambusia* to be ovoviviparous and described a number of embryological stages.

Zolotnitsky (1901), recording the habits of *Girardius decemmaculatus,* describes the mating and parturition, ascribing to the male the function of midwife; the male no doubt does show interest in the act but probably not from that motive. Zolotnitsky includes a series of observations on length of reproductive cycle and temperature. Philippi (1908) described in detail the gross and microscopic structure of both male and female reproductive tracts of two species of *Glaridichthys.* This paper is the most detailed one for any member of this family. As to the female reproductive system, he discusses the method of entrance of sperm into the follicle and of sperm storage, as well as the normal histology. Kuntz (1913), discussing the habits and the morphology of the reproductive organs of *Gambusia,* supplements and in a few places corrects Ryder's work. In general the embryology is shown to be almost identical with that of oviparous forms. Hildebrand (1917) carried on much the same sort of research, adding somewhat to the contributions of Ryder and of Kuntz. Milewski (1920) repeated much of

Philippi's work, and included also a number of other genera. He treats of the structure of the female and male organs (of which he distinguishes several types), mating, fertilization, parturition, sexual dimorphism, hybrids, etc.

These previous works are all from the point of view of the embryology and the anatomy of the reproductive tract. The subject may, however, be approached from the point of view of the ovarian cycle with its adaptations in their time relation to the cycle as a whole. The discussion resolves itself into two chief parts; first, a consideration of means of dating the pregnancies, and second, a description of the changes in the ovary and its contents during the cycle.

Material

Xiphophorus was selected because of its convenient size, hardiness in captivity, and apparently delicately poised sexual balance, as indicated by normal sex-reversals (Essenberg, 1923, 1926). The individuals used were mostly young breeding fish about 50 mm. in length and weighing about 1.5 grams. This size was preferred as the ovary is small enough for ready fixation while containing enough ova to allow material for weight and embryological observations to be taken without spoiling all of it for histological use. The offspring of large and small individuals do not differ noticeably in size, but the large females have larger litters than do the small ones.

Female Genital Tract

The ovary of *Xiphophorus* is a large unpaired body which in mature fish occupies most of the posterior part of the peritoneal cavity. Enveloping it is a thin, slightly pigmented peritoneal membrane. When this is removed the follicles or ovisacs appear lying quite separately, like grapes in a bunch, making up the bulk of the cortex. The follicle is composed of a single layer of cells supported by a thin theca, through which the contents are readily visible. Between the large follicles, with their golden amber ova, can be seen numbers of small white ova, usually located close to the central cavity and chiefly along its dorsal wall. These eggs form a continuous series from about 12 micra to 0.5 mm. in diameter. In shape they are commonly spheroidal but are often irregular, especially in preserved material. Close to the ovarian lumen is the conspicuous ovarian vein with small veins leading into it from a circular sinus on each follicle. These venous circles—described for *Gambusia* (Ryder, 1885)—are most distinct in the gravid ovary, becoming much less noticeable in the later stages of pregnancy. Penetrating to each ovum is a funnel-like invagination of the ovarian lining which widens out against the follicular epithelium at the bottom. This is called by Stuhlmann and by Philippi a *delle*. The sperm has access to the mature ovum at fertilization through a minute pore in the epithelium of the bottom of the delle. Later this opening enlarges to form the birth-pore through which the young escape into the central cavity at parturition.

Posteriorly, the ovarian cavity is continuous with that of the oviduct. This is not a homologue of the Müllerian duct, but is a single median tube arising from two primordia (Essenberg, 1923). One primordium is the posterior part of the ovary; the other the lining of the body cavity near the urogenital sinus. This oviduct is a short muscular tube elaborated into deep longitudinal folds, in the epithelium of which the sperm is stored, the sperm heads being visible within the high columnar cells. As the oviduct passes ventrally it widens and becomes less folded, then contracts sharply to an isthmus where the walls thicken to form a strong sphincter. Beyond this valve the oviduct again widens and runs to the tip of the genital papilla lying in the urogenital sinus which it enters from the anterior margin and very largely fills.

Procedure

A graded series of pregnant females was required from which other material could by comparison be assigned to definite stages. Data were taken on a random representative sample of fifty pregnant fish as to total length, length to end of scales on tail, greatest depth, depth of caudal peduncle, and body weight. The ovaries were opened and usually six ova or embryos removed. After measuring two or three representative embryos, three were dried, the others fixed in Bouin's with the remainder of the ovary.

The series was then arranged in order from the earliest to the latest stages of gestation. For the early stages, particularly those of growth of ova, the diameter of the egg was used as a criterion of developmental advancement; later the degree of differentiation of the embryo; finally the length of the embryo and size of the yolk sac. Now assuming the sample to be representative, the youngest stage should be post-parturient, which comparison with ovaries of known post-parturients showed to be true. Similarly the oldest stage should be pre-parturient—its ovary contained embryos indistinguishable from new-born fish—and the median fish should be halfway between, or should have passed through 50 per cent of the cycle. In this manner the fish were assigned percentage positions, so that the fifth fish was 10 per cent, the tenth 20 per cent, etc. This method of marking stages was deemed best because it represents a variable time interval which changes with the length of the gestational period. As under greenhouse conditions this period is about thirty days, 1 per cent equals about eight hours. For convenience the stages are indicated by the numeral alone, as, position 50.

Some justification perhaps is necessary for basing this curve on a sample of only fifty individuals. Undoubtedly it is to some extent inaccurate, but it appears to be sufficiently exact for practical use. First, there are no large gaps nor overlaps in the seriation—diameters and lengths follow smooth curves and show gradual increases. Second, several observations on fish kept under constant temperature conditions indicate much the same time relations. Third, the embryos of a litter are not all identical in size or degree of development, so that an appreciable overlap often exists between two adjacent series of young. For example, in a litter of new-born fish length varied from 8.6 mm. to 9.3 mm. As can be seen, a curve might be made more accurate than would be significant. Parturition, the only end-point for experimental timing, lasts a number of hours and may even be protracted over a period of several days.

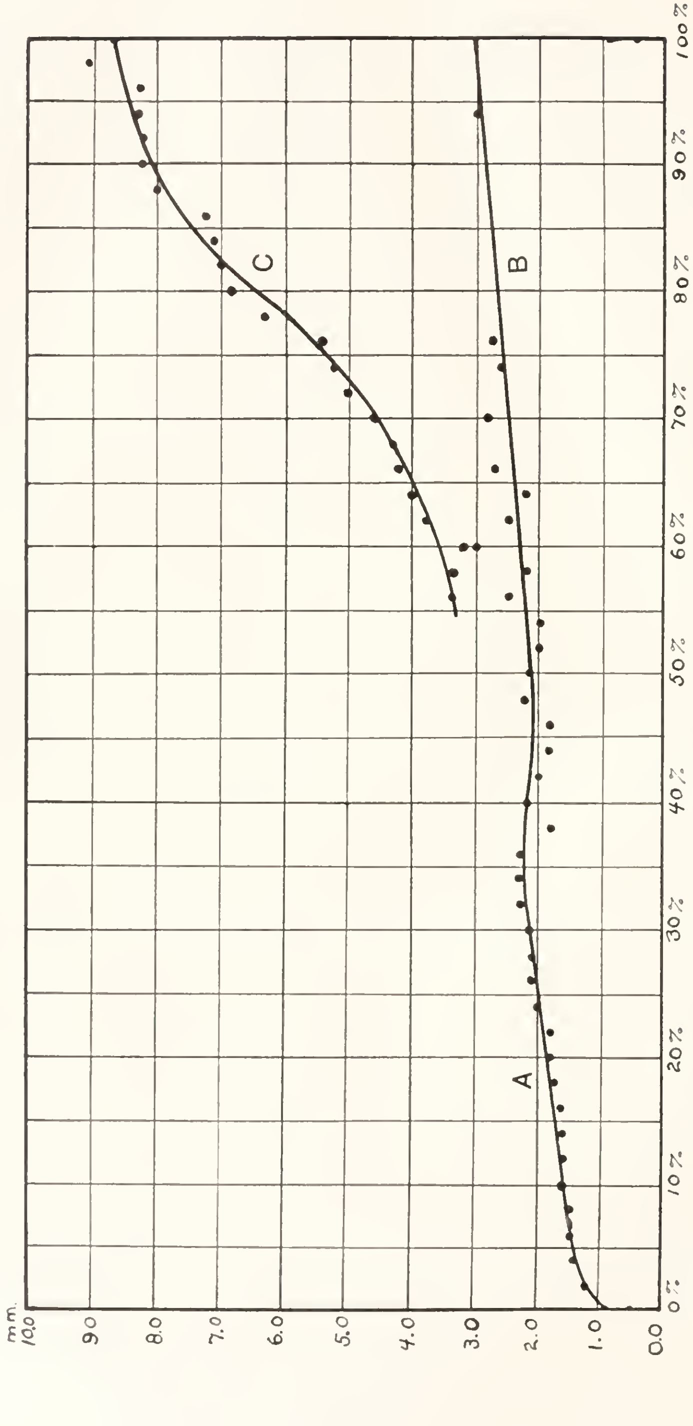
MEANS OF DATING PREGNANCIES

Ovaries may be assigned to relative positions in the cycle by comparison with a dated series, or more conveniently with Text Fig. 1, on which position is plotted against diameter of ovum, diameter of ovisac, and length of embryo, the chief criteria used in arranging the dated series. Between 30 and 50 differentiation of the embryo is very rapid and affords one a useful means for determining chronological position.

The desirability of a means of dating pregnancies by external criteria led to a study of body form and proportion using the ratio of greatest depth to depth of caudal peduncle and the weight length relations by means of the standard constant of conditions formula,

$$K = \frac{W}{L^3}.$$

The changes in proportion due to the stage of pregnancy were obscured by irregular variations ascribable to amount of body fat and to intestinal content. Consideration was also given to the "pregnancy spot" and to the scale markings. All four external criteria proved unreliable means of dating pregnancies, so that in practice the fishes had to be killed before their position could be ascertained. However, the "pregnancy spot" was found reliable as an indication of sexual maturity. Reproduction appears to play a comparatively constant rôle in the general physiology of the female *Xiphophorus*.



Text Fig. 1. Growth of the ovum, embryo, and ovisac; *A*—diameter of the ovum, *B*—diameter of ovisac, *C*—length of embryo.

The Yolk and the Follicle

A normal ovary contains, besides the group of large eggs, numerous smaller ones, ranging from ova with a diameter of 10 to 12 micra to large ones of about 0.5 mm. in diameter. Essenberg (1923) describes the origin of the definitive ova from the epithelial lining of the ovary, confirming Philippi's observation on *Glaridichthys*.

The smallest ovum with a follicle illustrated by Essenberg is one of 12 micra in which four or five follicular nuclei are seen around the ovum. The germinal vesicle is large and central. Fig. 1, Plate I, although a slightly older ovum, shows the same picture. The cytoplasm is quite strongly basophilic and the germinal vesicle is achromatic, showing two very chromatic nucleoli. The follicle is of very low squamous epithelium. As growth continues the cytoplasm becomes less basophilic (Fig. 2, Plate I). Here the germinal vesicle is even less chromatic than earlier, the only strongly basophilic portion being the large nucleolus. Around the low follicular epithelium the stroma is condensing to form a theca. The water content now must be very high as ova are often badly shrunken or collapsed if treatment is at all drastic. This is about as large as ova with homogeneous cytoplasm are found, for soon oil droplets appear in the borders of the cytoplasm. With the commencement of oil secretion, the epithelium begins to thicken, becoming cuboidal (Figs. 3 and 4, Plate I) and eventually moderately low columnar in type. Figure 4 shows a change in follicular epithelium with a rapid transition from cuboidal to columnar. The epithelium, which is high for follicles of this size, shows undulations as if to increase the surface; this is quite common. Philippi illustrates for *Glaridichthys* a series of follicular trabeculæ penetrating the yolk. My material does not show this except where it is an obvious artifact due to shrinkage or rupture of the ovum.

When a diameter of about 450 micra has been reached yolk secretion begins, a number of minute yolk granules appearing along one side of the ovum near the periphery. Those granules nearest the follicular epithelium are the most minute; those found more centrally are larger. Figures 5 and 6, Plate I, are of opposite sides of one ovum, and show a marked difference in the character of the epithelium and of the cytoplasmic inclusions. One side, Fig. 5, still contains only oil; the other, Fig. 6, both oil and yolk. As the yolk secretion continues, the entire mass of the ovum becomes filled with globules of oil and yolk. In histological preparations the oil is dissolved out, leaving cavities in the cytoplasm. The germinal vesicle still is achromatic and central in position. Such ova reach 0.5 mm. in diameter about parturition or immediately thereafter.

At this time these ova, which will form the next litter, are growing rapidly toward maturity. The follicle cells are cuboidal and very active. The ova are divided into two portions: centrally a structureless mass of coalesced yolk, peripheral to which is a layer of cytoplasm full of oil globules and yolk (Fig. 7, Plate I). This central yolk mass increases at the expense of the peripheral layer. The follicular epithelium is gradually reduced in height, probably due to two factors: (1) its physiological secretory function, and (2) the mechanical necessity for the single layer of cells to continue to cover the ever increasing surface of the ovum. At 30, after fertilization, the ovum and the follicle separate so that the ovum floats free in the cavity of the follicle which, from this point, is often called the ovisac (Fig. 8, Plate I). The ovum now is a mass of yolk with oil droplets embedded in its periphery. On one side is the blastodisc from which a thin membrane runs out onto the yolk. None of the material of this age shows a distinct vitelline membrane although one is found later surrounding the embryos. The follicle again consists of squamous epithelium supported by a vascular theca. As from this stage on the embryos and the follicle are not in contact, they are perhaps best discussed separately.

Throughout the period of gestation the epithelium of the follicle appears inactive, and becomes progressively thinner as does the theca, reaching an extreme of tenuity just before parturition (Fig. 9, Plate I, and Fig. 1, Plate II). Coincident with the development of the embryo there is an increase in the vascularity of the follicle.

At parturition the ovisac commences a new phase of activity (Text Fig. 3, *B*). As it collapses the squamous epithelium rapidly hypertrophies. The cells are columnar and are attached to each other only at the base (Fig. 2, Plate II). This strange epithelium has a windswept,

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Explanation of Plate I

Camera lucida drawings from Delafield's hæmatoxylin and eosin preparations. × 453. *Y*—yolk, *T*—theca, *O*—oil, *F*—follicular epithelium, *P*—peritoneum, *S*—secretion.

Fig. 1. An early ovum showing germinal vesicle nucleoli, and follicle.

Fig. 2. An older ovum showing follicle with a theca.

Fig. 3. An ovum, average diameter 200 micra, showing beginning of oil secretion.

Fig. 4. An ovum, average diameter 310 micra, showing transition from cuboidal to columnar epithelium.

Fig. 5. An ovum, average diameter 450 micra, showing late stage of oil secretion.

Fig. 6. The opposite side of the same ovum, showing the commencement of yolk secretion.

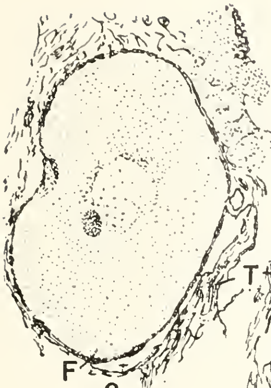
Fig. 7. An ovum at 10, showing yolk secretion at its height.

Fig. 8. An ovum at fertilization—the cessation of yolk secretion.

Fig. 9. Wall of an ovisac at 100, showing its vascularity.

Fig. 10. Wall of a spent follicle, showing it full of secretory material.

Plate I



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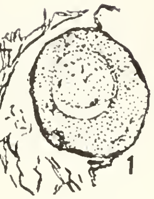

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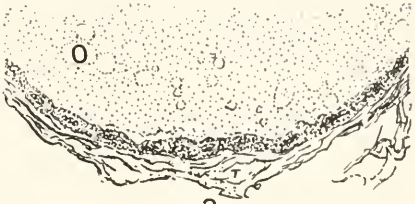

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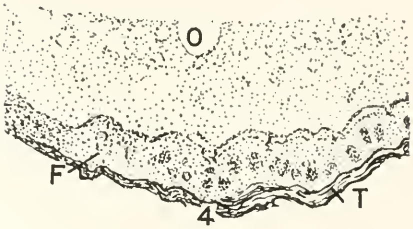

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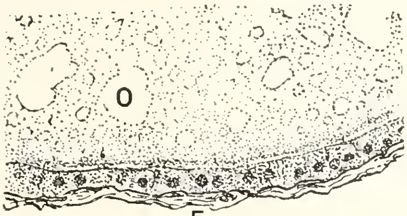

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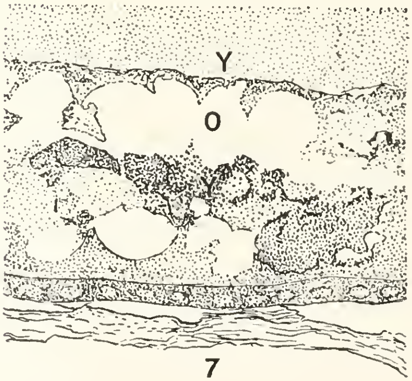

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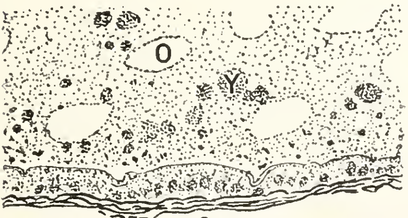

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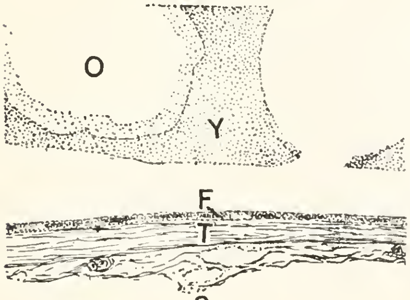

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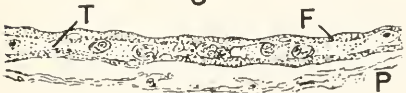

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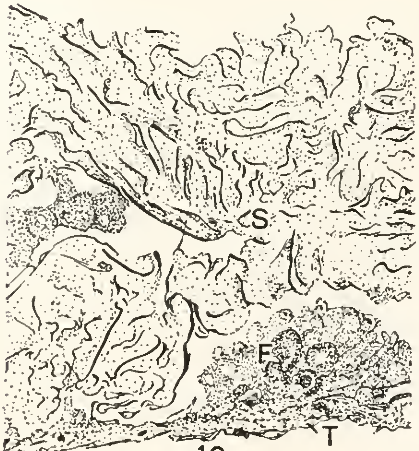

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fringed appearance. The nuclei are achromatic and are found well up from the base as if to indicate considerable activity. Beneath the follicular epithelium, the theca has also greatly thickened and bears numbers of polygonal cells. The blood vessels also are enlarged and form a zone in the thickness of the follicle wall (Fig. 3, Plate II). The follicle then begins to secrete, Fig. 4, Plate II, and to slough, so that its lumen is filled with the secretory product, which resembles a mass of crumpled membranes with here and there some cell fragments. This secretion passes into the lumen of the ovary (Fig. 10, Plate I, and Fig. 5, Plate II).

Vitelline Plug

Within the vitelline membrane of ova at about position 70, a plug is to be found closing the birth-pore. This is composed of a mass of vesicular material resembling in structure the cytoplasm of ova prior to the commencement of yolk secretion (Text Fig. 3, *A*). This mass is found just opposite the birth-pore as if it had been first loose and then settled in the slight bulge where the vitelline membrane bellied out into the lumen of the ovary. As to its nature, it may be any of three things, i.e., nutrient material from the parent, excretory product of the embryo, or a secreted reinforcement for the membrane across the birth-pore. This last seems the least probable suggestion, as this membrane in *Xiphophorus* is really only a functionless rudiment. The plug seems rather too firm and solid to be an excretory product; moreover, the embryo has no fecal material in the gut and since no goblet cells are present as yet, the plug is not apt to be mucous in nature. It seems most likely to be either a nutritious substance newly secreted from the follicles, as in *Anableps,* or modified yolk cut off from the yolk mass prior to its complete enclosure by the embryo.

Structural Changes in the Embryos

The embryology, as before mentioned, has been described in this family. The various authors agree that the development of viviparous forms differs in no significant particulars from that of related ovi-

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Explanation of Plate II

Figures 1 to 4, × 320. Fig. 5, × 72.

Fig. 1. A section showing relation of embryo and follicle just prior to parturition. *Y*—yolk in yolk-sac of embryo, *Ys*—yolk-sac, *V*—vitelline membrane, *F*—follicle.

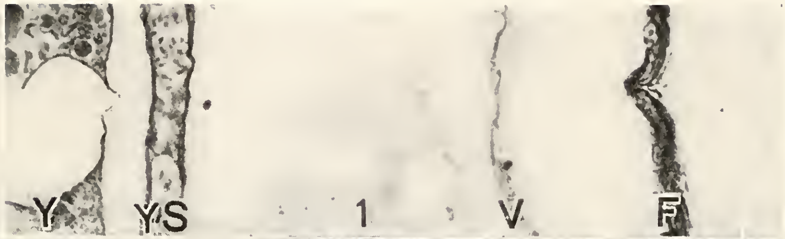
Fig. 2. Wall of collapsed follicle showing hypertrophied theca and the follicular cells.

Fig. 3. A slightly later stage showing the zone of capillaries.

Fig. 4. The follicle just beginning to secrete.

Fig. 5. A follicle at the height of secretion.

Plate II



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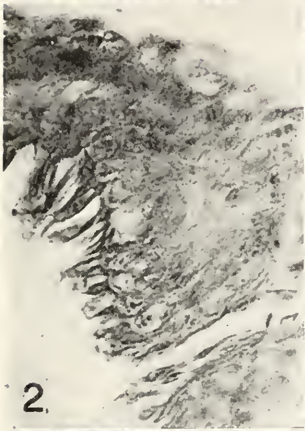

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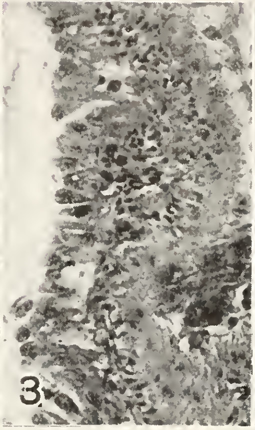

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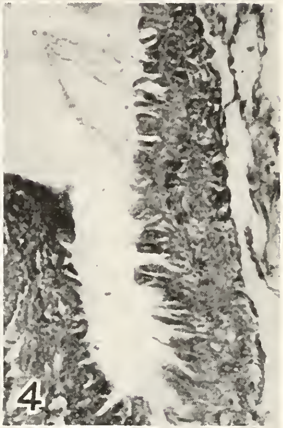

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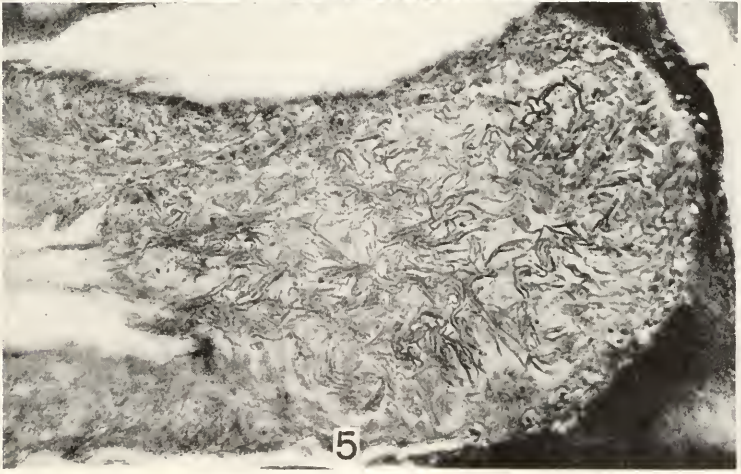

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parous species. This section does not purport in any way to describe the embryology or to give a complete picture of any stage or any system, but to present such conspicuous characters as would be of practical use in determining the age of the material, especially in sections of the ovary where the embryos may be cut through any plane. Fertilization stages have not been found but they occur at about 30. At this time the ovum has reached a diameter of 2.1 mm. and consists, except for the blastodisc, of a solid ball of yolk with numerous large oil drops around the periphery. Fertilization does not seem to take place simultaneously in all of a batch of ova, so that different stages of cleavage can be observed in the same ovary. Figure 3, Plate III, shows one of the blastodiscs from an ovum at 30, which is composed of about 60 cells.

At 40 (Fig. 4, Plate III) the blastodisc has spread and formed the germ ring on one side of which the embryonic shield has just appeared. The shield lengthens and development is rapid so that at position 50 the embryo is well formed.

The embryo (Fig. 5, Plate III) is about 2 mm. long and has already produced an excellent vitelline circulation. Well-formed optic cups with lenses in them are visible externally. The cup is differentiated into retina and choroid coats and the choroid fissure still remains. Somewhat posteriorly the large spheroidal otic vesicles appear. The lateral ventricles of the brain are beginning to grow and the myelencephalon shows its characteristic thin dorsal wall, although the nervous tissue is not as yet differentiated into white and gray substance. One also may note that the anterior neuropore is open, the tail is free on the yolk, and that there are still no limb buds. The notochord is differentiated in the posterior part.

At 60 the embryo has reached a length of about 3.6 mm. (Fig. 6, Plate III). The embryo has proportionately a very large head to which

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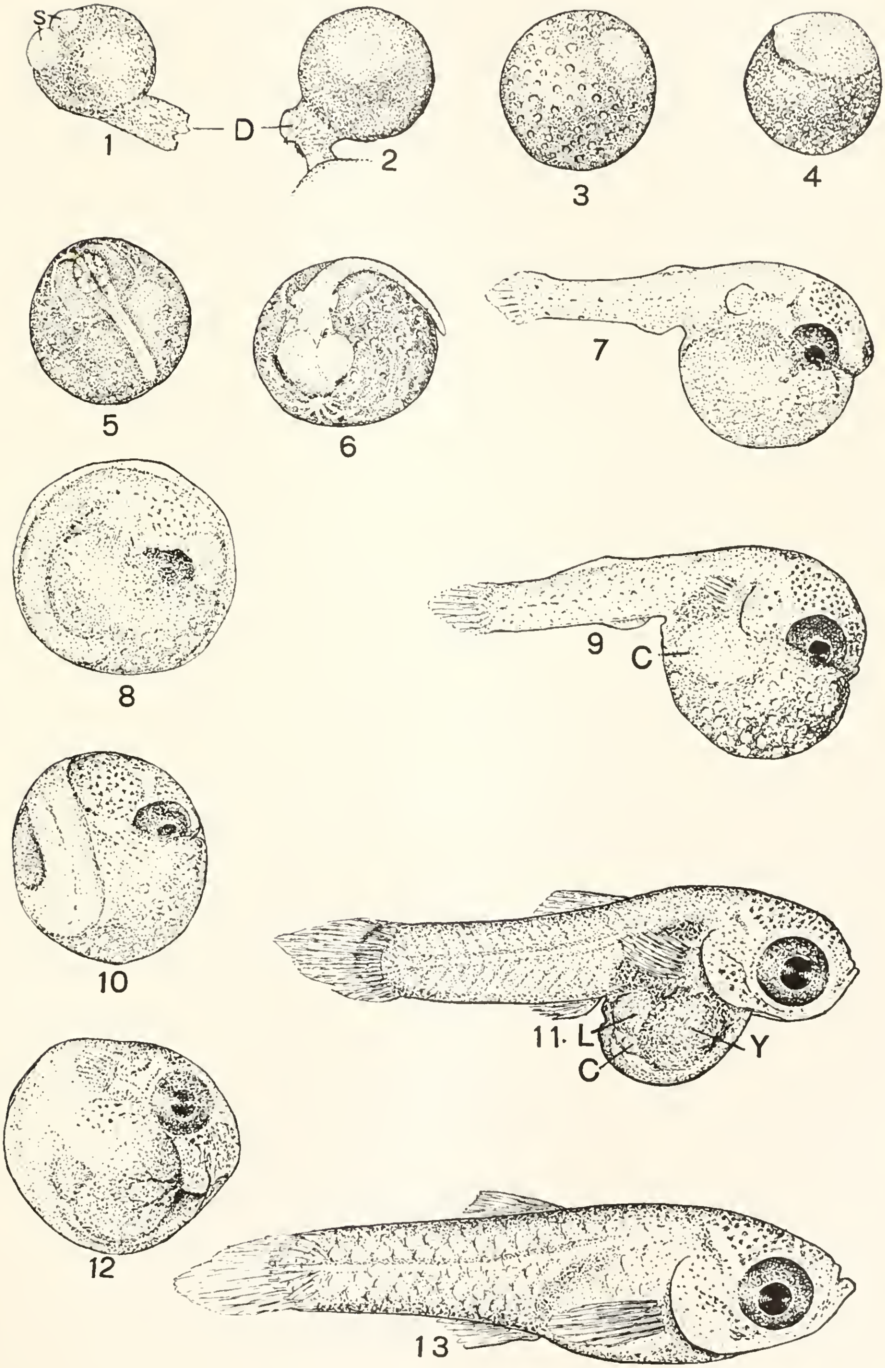
Explanation of Plate III

Camera lucida drawing × 11.25. Embryos fixed in Bouin's and cleared in alcohol and glycerine.

FIG. 1. Ovum in follicle at 10. *S*—small ovum in theca of large one, *D*—attachment to center of ovary; this stalk contains a delle.
FIG. 2. Ovum in follicle at 20.
FIG. 3. Ovum at 30 showing a late cleavage stage.
FIG. 4. Ovum at 40 showing an early embryonic shield.
FIG. 5. Embryo at 50.
FIG. 6. Embryo at 60, showing pigmented eyes and limb buds.
FIG. 7. Embryo at 70.
FIG. 8. Embryo at 70 enclosed in the ovisac.
FIG. 9. Embryo at 80, *C*—cœlom.
FIG. 10. Embryo at 80 in its customary position.
FIG. 11. Embryo at 90, *Y*—yolk, *C*—cœlom, *L*—liver.
FIG. 12. Embryo at 90, coiled in the ovisac.
FIG. 13. Young at birth.

Plate III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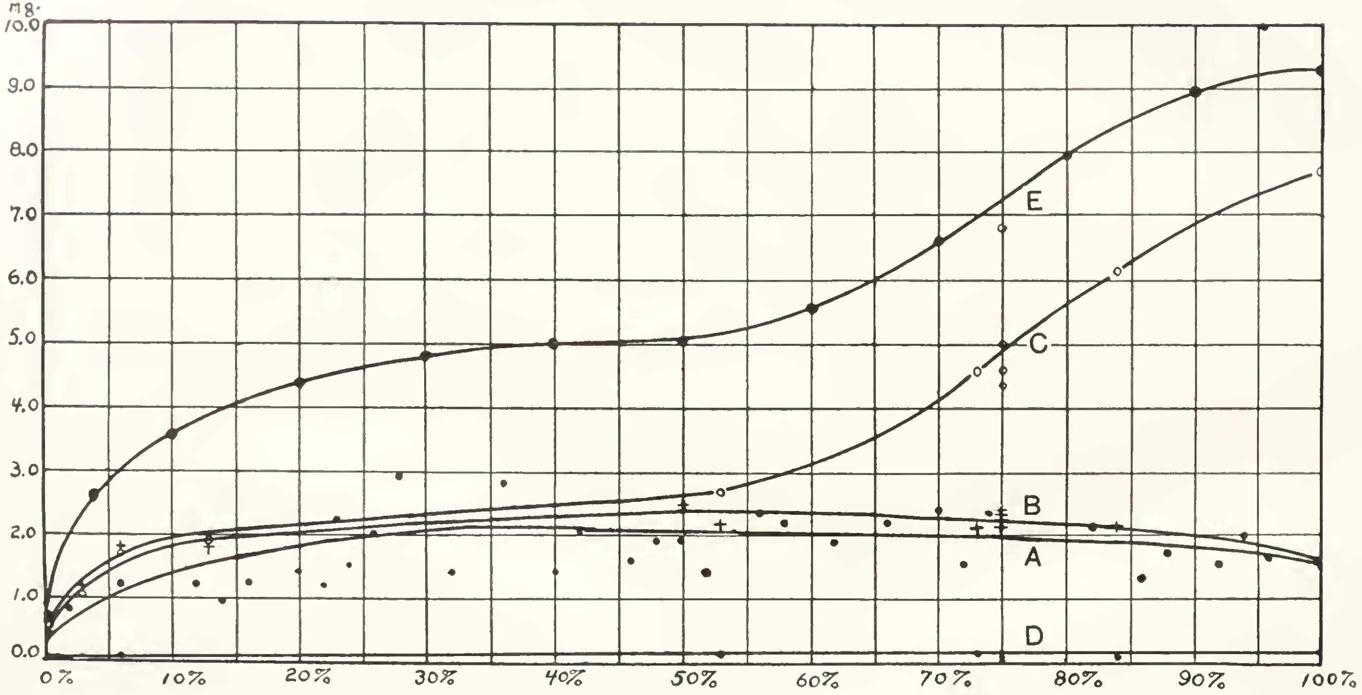
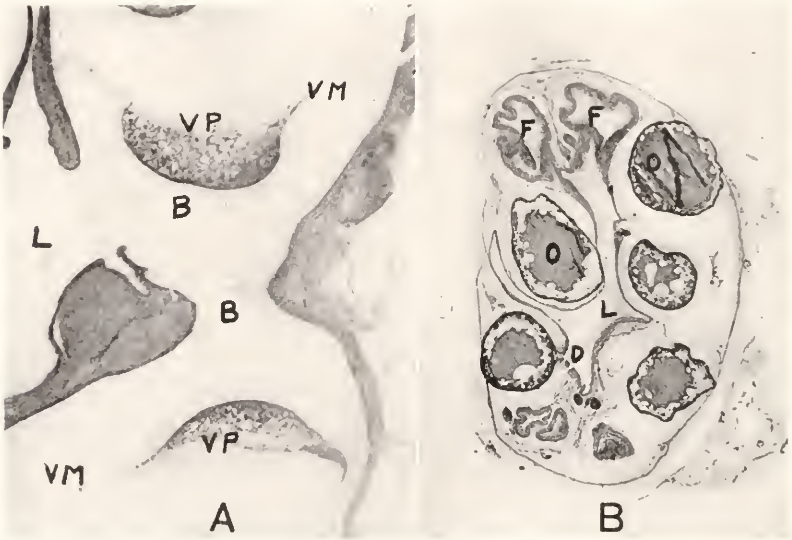


TEXT FIG. 2. Composition of ova and embryos; *A*—dry weight from dated series, *B*—organic content, *C*—water, *D*—ash, *E*—total weight, i.e., total of *B*, *C*, and *D*.

the trunk, with its newly acquired limb buds, appears as an appendage. The eye shows considerable advance, pigmentation being in progress. The lens is fibrous and the retina is separating medially into the ganglionic and nuclear layers. Elsewhere the nervous tissue is differentiated into gray and white substance, and a number of nerves have appeared. The beginning of histogenesis of cartilage and muscle occurs at about this time. Development of the digestive tract has resulted in a flattened pharynx lined with cubical epithelium and a tubular gut lined with columnar epithelium. Filling a considerable part of the cœlom is the liver anlage, a highly vascularized mass of rather darkly-staining polygonal cells. Another new organ is the pronephros, which here is only a slightly bent tubule just posterior to the pharynx.

Figures 7 and 8, Plate III, show the general appearance of an embryo at 70 in the ovisac, and released. At this stage, 4.6 mm., the head, although large, does not overwhelm the body but seems to fit it. The snout tends to be buried in the yolk up to the eyes or above. Borne upon the trunk, which usually is curled slightly to the side, are dorsal, ventral, and caudal fins. Only in the caudal fin are any fin rays to be found although the cartilages of the pectorals are formed. Pigmentation has now progressed so that chromatophores are abundant over the cerebrum and are occasionally to be seen on the caudal peduncle. Internally the peritoneum is also pigmented. Sections show the olfactory anlage to be still only thickened placodes; while the otic vesicles have developed and are now no longer round but irregular, due to the marking out of the semicircular canals. A pair of thin cartilaginous walls rises one on either side from the otic capsules to protect the brain. The spinal cord is protected similarly as there are cartilaginous lamellæ in the neural arches of the trunk and in the hæmal arches of the tail; these latter, however, are very minute. In the pharyngeal region the thyroid anlage forms a pit in the floor of the pharynx. On either side are the visceral pouches, which so far contain no gills. Curled under these and mostly out of sight are the operculæ. The pineal body and the pituitary are now well developed.

Figure 9, Plate III, shows an embryo at 80 free, and Fig. 10, a littermate in the position customarily taken in the ovisac, with the tail flexed and held over the back of the head. The snout is slightly embedded in the yolk. The pigment cells are distinct along the lateral line of the tail. Within the posterior part of the yolk sac is a clear vesicle, the enlarged cœlom. Dermal fin rays have now appeared in the pectoral fins. In the region of the otic vesicles the cranium is beginning to roof over the brain. The ribs are now present. Pharyngeal teeth are found although so far but little dentine has been deposited. The olfactory apparatus is a deep flask-shaped pit with a thickened sensory epithelium at the base. Thickened sensory areas are also to be found in the lining of the canals and ampullæ of the ear. A cross-section of the pharynx shows distinct gill bars which, with the exception of the last, bear no filaments. Medial to the gill bars along the ventral aorta the thyroid appears, consisting of scattered follicles full of colloid. The viscera have changed somewhat; the gall bladder which earlier was lined with cuboidal cells now has a squamous lining; distinct pancreatic rudiments are present in the mesenteries. The cells of the gas gland have become large and achromatic.



Text Fig. 3. *A*. Section showing vitelline plugs closing the birth-pores. × 72. *L*—lumen, *Vp*—vitelline plug, *Vm*—vitelline membrane, *B*—birth-pore. *B*. Section of an ovary at 10 × .15. *O*—ovum, *F*—empty follicle, *D*—delle.

At 90 the *Xiphophorus* embryo is so long that in the ovisac the animal forms a complete loop, keeping its tail on the anterior part of the head. When released it straightens out to about 8.1 mm. (Figs. 11 and 12, Plate III). The yolk sac is an irregular mass which in preserved material shows a number of distinct parts. Anteriorly are the heart and vitelline veins, then the yolk proper which as an almost bipartite mass occupies ventral and lateral positions. In the notch between the halves of the yolk are two swellings, the liver and the cœlom. The operculæ now are conspicuous as they stand out from the head and are studded with many large chromatophores. The dorsal and anal fins have fin rays as well as the pectorals and caudals. The pelvic fins are represented only by thickenings just anterior to the anus. A section through the optic chiasma shows large lymph sinuses in the orbit and between the eyes. Below in the lower jaw is a tongue cartilage which forms a distinct inverted U. A sheath is now being laid down upon the notochord. In the body cavity changes are slight; the kidneys are much more vascular than before and show the capsules around the glomeruli. The lining of the stomach and intestine is thrown into folds sparingly supplied with goblet cells.

At parturition (Fig. 13, Plate III), we find a perfectly formed little fish about 8.9 mm. long, short and plump, with a smoothly rounded belly, quite capable of looking after himself and ready to feed or to dodge his cannibalistic relatives. Recently he has acquired a set of scales and greatly elongated his pectoral fins. The retraction of the yolk seems to be accomplished chiefly by the removal of cœlomic fluid, the place of which is taken by the yolk and liver which, up to this point, have projected far below the normal body contour. The yolk now surrounds the truncus arteriosus on three sides, lying dorsal to the heart and between the ducts of Cuvier, extending throughout and filling almost the entire body cavity. The stomach, intestines, and skin are copiously supplied with goblet cells.

Weight and Composition of Ova and Embryos

In the course of development, changes take place not only in form and differentiation but also in weight and composition of the ova and embryos. Text Fig. 2 shows the results of two sets of weight measurements. *A* represents data derived from the dried embryos from the dated series. These were air-dried upon cover-slips, weighed on the slip, then removed by scraping and the weight of the cover-slip determined. Weights so obtained were relative but not exact as the degree of desiccation and the completeness of removal from the slip were variable. The slight residue left on the slide makes this curve uniformly low. Curve *B* presents data derived from a special series where quantitative determinations of organic content were made; curve *C* for measurements of water content; curve *D* for ash; and curve *E* for total weight. The number of individuals used for the determinations was governed by the number which could be dissected intact from the ovary, i.e., eight to twenty-five.

The most interesting of these curves have to do with the dry weight of the embryo and its parallel, the organic content of the embryo. These two curves are so similar that they may be discussed as one. The ova are shown to increase in weight until maturity, then upon fertilization

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at 30 the increase gradually ceases and a slight decline begins. This is ascribable to the utilization of the yolk in the normal metabolism of the embryo. The two curves differ slightly in that *A* is fitted most perfectly by an almost straight line from 30 to 100 while from 70 to 100 *B* fits a more strongly curved one, indicating a slight increase in rate of yolk consumption. *B* appears more reliable than *A* as the weights were determined with greater accuracy. If *D*, the ash content, were added to *B*, it would then be exactly comparable to *A* and would more nearly show the same straight line picture.

The data on ash content are not of much significance, being almost within the error of the determination. At a maximum it is but a little over 0.1 mg. per embryo. It does, however, suggest that, unlike organic content, mineral salts continue to increase in quantity after fertilization, being taken up as needed by the embryo.

As the yolk decreases and the ash content is small, the obvious increase in volume of the embryo is seen to be due to absorption of water. During the early stages of egg development, the percentage of water is high as small ova shrink easily and are distorted in many sections. As the deposition of oil and yolk commences, the organic material accumulates faster than does the water so that water content falls nearly to 50 per cent, which it approximates until 60. When yolk ceases to be laid down, the percentage of water is increased by continued absorption. This absorption continues at a rate of increase corresponding to the growth of the embryo, the water content reaching at birth about 84 per cent of the total weight. Total weight represented upon Text Fig. 2 by Curve *E*, which is the summation of *B*, *C*, and *D*, shows rapid increase until fertilization—the period of yolk secretion—then, a pause followed by a notable increase—the period of water absorption.

DISCUSSION

While ordinarily pregnancies are readily classified as either viviparous or ovoviviparous, it is not always easy in practice to determine the nutritive status of the embryos without obtaining their dry weights at various stages.

In several families, Embiotocidæ and Zoarcidæ, the young feed upon a cellular secretion or ovarian milk but the presence of material within the follicles or the lumen does not seem entirely diagnostic of true viviparity. *Xiphophorus*, a distinctly ovoviviparous species, has material within the vitelline membrane—the vitelline plug. As the exact nature of this material is unknown, little can be said other than that suspicion might be thrown upon a claim concerning the nutritive status of a teleost embryo, where dependence was placed upon this criterion alone.

Absence of special organs of absorption in the embryo does not necessarily indicate the ovoviviparous condition; any body surface, if somewhat permeable, may function as a pseudo-placenta. In the pœcilids the condition of *Anableps*, in which the young are retained and nourished in the ovarian lumen, can be derived from that of *Xiphophorus* by two relatively minor changes: (1) a continued secretion by the follicular cells after their separation from the ovum, and (2) some membrane of the embryo remaining permeable to certain large molecules such as those of fatty acids or amino-acids.

The anatomy of the ovary of *Xiphophorus* shows an almost schematically perfect nutritive relationship between parent and offspring. Within the peritoneal investment is a loose œdematous stroma, practically a lymph cavity, in which the follicles lie and from which the young ova derive nourishment before the establishment of their follicular circulation. The blood in the follicular capillaries seems to be separated from the cavity only by the endothelium and the squamous follicular epithelium. The embryo floats free in the follicular cavity, surrounded only by a very thin vitelline membrane. Beneath the epidermis of the yolk-sac is a dense bed of large blood vessels which function in the absorption of the yolk. This arrangement requires a molecule to penetrate four layers of cells and the vitelline membrane in passing from the blood stream of the parent to that of the embryo, a separation similar to that found in many mammalian placentas. Apparently *Xiphophorus* fails to become truly viviparous because of physiological rather than anatomical inadequacies.

The spent follicle of the teleosts is at times compared with the corpus luteum of the mammalia and is even so called by some authors. It is customary to limit the term to such ovulated follicles as contain that particular sort of irregular secretory cells known as lutein cells.

Cunningham (1897) describes the spent follicles of the plaice and the gray gurnard, both oviparous species. He finds the follicular epithelium granular and separated from the theca; it shows no signs of hypertrophy or proliferation. In late stages the follicle appears as a mass of connective tissue, which he thinks is most probably the much shrunken theca. No mention is made of any secretory activity of the follicle. But in the case of the weever, it is said to be full of cellular tissue. Wallace (1903), describing the spent follicle of the viviparous blenny, *Zoarces*, states that on rupturing the follicle collapses and becomes a papilla with only slight hypertrophy of the follicular epithelium. This papilla, according to Stuhlmann (1887), secretes the ovarian milk on which the embryos feed.

In *Xiphophorus* we find a hypertrophy of the spent follicle and of the theca. The enlarged follicular cells do not look like lutein cells but much more like certain endometrial cells than like anything to be found in mammalian ovaries. The bulky secretion, which fills the follicle, cannot be of present nutritive use as the embryos are already delivered and the new follicles are not yet open to the lumen. It is unlikely that this represents only a retrogression of the follicle as it has not been described for oviparous teleosts nor does the follicle of disintegrating ova go through this phase. It conceivably might serve an endocrine function or serve to activate the sperm stored in the cells of the oviduct. In general *Xiphophorus* agrees with other teleosts in that its spent follicles cannot properly be termed corpora lutea.

Conclusions and Resumé

1. *Xiphophorus* is, in the anatomy of its female reproductive tract, very similar to *Gambusia* and *Glaridichthys.*

2. External dating of pregnancies is difficult, as body proportions, markings, and scale rings were found to be non-diagnostic.

3. The diameter of ova and length of embryos serve effectively to date ovaries which have been dissected.

4. Ovaries can also be assigned approximately to position from sections by noting the degree of differentiation of organs and tissues of the embryos.

5. Ova of varying size and yolk content can be found in the normal ovary at any stage of the cycle and are hence of no value in dating pregnancies.

6. As yolk accumulation commences, the height of the follicular cells decreases.

7. The follicular epithelium of a young ovum reaches its greatest height during the period of oil secretion before yolk granules appear.

8. After parturition the spent follicles collapse and hypertrophy, forming thick thecæ with a zone of capillaries and superficially a layer of tall columnar cells attached to each other only at the base.

9. The hypertrophied follicle, which is not considered directly comparable with the corpus luteum of the mammals, secretes and sloughs. The secretion appears as a crumpled mass of membranes and passes into the lumen of the ovary.

10. The organic content increases in the ovum until fertilization. From there on it becomes steadily less as it is used up by the growing embryo.

11. The ash content appears to increase throughout the entire period of gestation.

12. Water is absorbed in ever increasing amounts throughout the entire period of growth of ovum and embryo.

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THE ACTION OF THE CRUSTACEAN CHROMATOPHORE ACTIVATOR ON THE MELANOPHORES OF FISHES AND AMPHIBIANS

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In addition to inducing color changes in the crustaceans, the chromatophore activator of the crustacean eye-stalk also brings about contraction of fish melanophores (Koller and Meyer, 1930; Meyer, 1931), and expansion of amphibian melanophores (Perkins and Kropp, 1932). The range of occurrence and effectiveness of the eye hormone is very wide among the crustaceans, undoubtedly extending throughout the class where active chromatophores are present in the larval or adult stages (Kropp and Perkins, 1933). Concerning the action of the crustacean eye hormone on the melanophores of fishes, it is of interest to know the extent to which this substance is effective and the nature of the response by the fish melanophores. For this purpose several fishes were selected as representative of the local fauna, and sea-water extracts of various decapod eye-stalks were injected into them. The procedure consisted of adapting the fishes to white and to black backgrounds, noting the general coloring of the fish and the microscopic appearance of the melanophores. Measured amounts of the extract were injected into white-adapted and into black-adapted specimens and the fishes returned to their original black or white containers. Control animals in each group received a like injection of sea water.

The injections were made for the most part subcutaneously just ventral to the lateral line at the middle of the body, although in some cases it was given directly into the blood stream. Since no assay of the active substance that will permit the use of doses of known or comparable strength has yet been made, the procedure tentatively adopted was that previously outlined (Kropp and Perkins, 1933) of making the quantity of extract injected equivalent to a known number of eye-stalks. Two hundred eye-stalks were macerated in 1 cc. of sea water, boiled, centrifuged and the clear extract decanted off. The latter was then diluted with sea water to 2 cc., thus giving a solution in which each 0.01 cc. contained the active material of one eye-stalk.

RESULTS

Anguilla rostrata

When about fifteen centimeters long this eel shows a large number of discrete melanophores that are easily observable with low magnification. It shows also the usual reactions to backgrounds,—melanophore expansion on black and contraction resulting in pallor on white backgrounds. The reaction, however, is relatively slow, for freshly captured animals may take as long as ten hours to show the characteristic white-adapted or black-adapted state, although some specimens may adapt in half the time. When they can be induced to remain motionless the rate of adaptation proceeds more speedily. The eels' response to extracts from the eye-stalks of white-adapted *Crago* was by no means sharp. Injections were made both subcutaneously and directly into the heart in different animals without any detectable difference in response. The minimum effective dose was 0.04 cc. of extract representing four eye-stalks. There were no lasting ill effects in most cases, but the eels never survived more than twenty hours if the quantity of fluid injected was increased to 0.05 cc. although uninjected animals could be maintained in the laboratory for indefinite periods.

At best there was a weak though definite response of the melanophores to the injection. In three to five minutes after injection, black-adapted eels showed a slight general melanophore contraction, somewhat more marked in the region around the place of injection. There was no apparent difference between animals receiving the injection subcutaneously and those receiving it directly in the blood stream. The effect obtained was always very transient and after ten to fifteen minutes the animals reverted to their original states.

Clupea harengus

In the black-adapted state the herring presents a fairly uniform dark metallic blue over the entire dorsum and sides. In the white-adapted state the blue gives way to a deep brown. We were not able to investigate the pigmentary conditions actually existing in the skin although it is apparent that some of the color phenomena that appear are due to interference. The changes from one state to another take place rapidly and never require more than one-half to one hour. The venter always retains its silvery white condition.

The minimum dose inducing an effect was ten eye-stalks (0.4 cc. of fluid used). In a black-adapted herring the effect of the extract was to bring on a local change at the place of injection. This change took the form of intensifying the blue characteristic of the black-adapted state.

It occurred within five minutes after the injection and after ten minutes this effect disappeared and no general effect was ever noted. In white-adapted animals, however, thirty minutes after the extract was injected the entire dorsum of the animal was blue-green and approximated closely the black-adapted state. The controls were injected with sea water, the black-adapted controls showing no effect, while the white-adapted controls took on a mottled blue-green state after one hour. Although the responses of both experimental and control white-adapted animals were alike in many respects, there were some striking differences,—namely, the slowness of the reaction in controls, one hour compared to one-half hour in the extract-injected animals, and the presence in controls of a pronounced mottling and never the uniform dark blue-green of the experimental animals. These differences in reaction are regarded as due to the presence of the chromatophore activator in the experimental group. Blue and blue-green color of sides and dorsum are assumed to be associated with a condition of melanophore expansion.

Pollachius virens

The ability of the pollock to adapt to its background is not highly developed. After several hours on a white background there was a suggestion of pallor over the back and sides, but marked differences in pigmentation were never observed. The injection of the extract also yielded no changes either in gross coloration or in the microscopic appearance of the melanophores. Doses of twenty eye-stalks failed to induce an effect in either black-adapted or white-adapted animals, whether the injections were made subcutaneously or by way of the caudal artery.

Scomber scombrus

The mackerel, unlike the pollock, shows a marked difference between the white-adapted and black-adapted states. In the former, black barring of back and sides is sharp and the spaces between bars are pale green-blue. In the latter, the intermediate spaces are blue-black to dark green or gray-black. The change from one condition to the other occurs readily in fifteen to twenty minutes. Like the pollock, however, this fish did not react to injections of extracts of as many as twenty eye-stalks.

Fundulus heteroclitus

For the minnow, *Fundulus,* extracts from eyes of *Homarus americanus* and *Pagurus longicarpus* were used in place of that from *Crago.* Mere puncture of the skin and underlying muscle while making an injection usually resulted in the formation of a local spot of expanded melanophores due to the stimulation of a peripheral pigment motor nerve. Darkening due to such a stimulus, however, could always be detected promptly by the characteristic rapidity with which the effect occurred,—it was almost instantaneous. When a black-adapted *Fundulus* was injected with an extract of ten eyes of either *Homarus* or *Pagurus,* there usually occurred first the just mentioned local area of expanded melanophores. After five to fifteen minutes there supervened a condition in which the melanophores of this area contracted gradually until they were maximally contracted.

When a needle was simply thrust through the skin and muscle but no extract injected the same melanophore expansion resulted locally, but this lasted for five to twenty hours before again assuming the condition of the other body melanophores. The rapid contraction of the expanded melanophores of this region is to be ascribed, therefore, to the ability of the crustacean eye hormone to overcome the counter tendency of mechanico-nervous stimulation.

The area of contracted melanophores increased somewhat in size depending on the strength of extract injected. General pallor, however, was brought about only by means of a large dose which also induced serious ill effects and, in most cases, death. In one instance where an extract of twenty *Pagurus* eye-stalks was injected into a black-adapted *Fundulus* evidence of pallor first appeared after ten minutes. The pale patch at the place of injection then increased rapidly in size and the fish at the same time showed pronounced ill effects of the extract. It gasped, lay on its side, and its movements were distinctly abnormal. It survived for some fifteen hours after the injection, and during that time the pale area was extended to include the entire animal except for the dorsal and lateral surfaces of the head which remained black. In sub-lethal doses, then, the effect was to produce an area of contracted melanophores at the place of injection.

Before the first melanophore expansion due to nerve stimulation wore off there occurred normally a sharp melanophore contraction in the center of the area which spread peripherally until most of the melanophores in the entire area were contracted. It occurred in both black-adapted and white-adapted animals but was best seen in the latter. Here, the puncture produced an initial darkening, which soon gave way to pallor until there remained only a narrow band of expanded melanophores surrounding a region of maximally contracted ones. In most cases the contracted melanophores remained so for about two hours, but the area did not increase in size after the first fifteen minutes. With doses approaching the lethal the just described effect was produced but the reacting melanophores then failed to revert to their expanded or stellate condition and acted subsequently as though paralyzed, and in some cases were known to have been killed.

Myoxocephalus octodecimspinosus

The long horn sculpin adapts very readily to backgrounds and also tends to adapt to the pattern of its background after the fashion of flat fishes. Injection of extract of ten eye-stalks of *Crago* into both black-adapted and white-adapted animals resulted in increased pallor all over the body. The marked local reactions characteristic of other species were here lacking in most specimens. The reaction set in five minutes after injection and disappeared after one hour. During the time that the extract was effective there was complete loss of ability to adapt to background and to pattern. The controls showed no response.

Limanda ferruginea

While the common dab adapts to backgrounds and to background patterns, it does not do so with the same ease and speed characteristic of other flat fishes. *Limanda* was able to survive a maximum dose of fifteen *Crago* eyes, and ten to fifteen minutes after the injection both black- and white-adapted animals exhibited a weak tendency towards melanophore contraction at widely separated parts of the body, but especially at the place of injection. This is in line with the results obtained by Miss Meyer (1931) with extracts of *Crangon* eyes on *Pleuronectes,* but she describes a more marked local reaction than we were able to obtain. It may well be, however, that the difference in results is due to a quantitatively inferior pigmentary reaction in *Limanda.* Unlike *Myoxocephalus,* the extract did not produce a loss of ability on the part of *Limanda* to adapt to its background pattern.

Rana clamitans

Previously the tadpoles of this frog were described as responding to injection of eye-stalk extracts from *Palæmonetes vulgaris* and *Callinectes sapidus* by melanophore expansion (Perkins and Kropp, 1932). In repeating those experiments with extracts from *Pagurus longicarpus* and *Homarus americanus* effects identical with those previously described were obtained. Extract of two eyes of either of these crustaceans was sufficient to induce melanophore expansion in white-adapted tadpoles five minutes after injection. Maximum expansion was reached after twenty to thirty minutes. Contraction began immediately and the original white-adapted state was reached again one hour after the injection.

It was very striking to find, as we had in the case of *Callinectes,* that decapods which do not ordinarily show adaptive color changes or any chromatophore movements at all in response to backgrounds (*Homarus, Libinia, Pagurus*) all possess the active principle in their eye stalks. This is possibly a retention in the adult of a mechanism which is active in the larva where the chromatophores are mobile. The hormone of such crustaceans was found to be effective in producing chromatophore movements not only in other crustaceans, but, as shown in the present work, in fishes and amphibians as well.

TABLE I

Species receiving injection	Eye-Stalk Extract	
	Black-adapted	White-adapted
Anguilla rostrata	Slight contraction	No response or slight contraction
Clupea harengus	No response or doubtful expansion	Moderate expansion
Pollachius virens	No response	No response
Scomber scombrus	No response	No response
Fundulus heteroclitus	Extreme contraction	Moderate contraction
Myoxocephalus octodecimspinosus	Moderate contraction	Moderate contraction
Limanda ferruginea	Slight contraction	No response or slight contraction
Rana clamitans (tadpoles)	Moderate expansion	Extreme expansion

The extracts used were from eye-stalks of white-adapted *Crago vulgaris* in the cases of *Anguilla, Clupea, Pollachius, Myoxocephalus,* and *Limanda;* from *Pagurus longicarpus* and *Homarus americanus* in the cases of *Fundulus* and *Rana.* The terms "black-adapted" and "white-adapted" refer to the condition of the animal receiving the injected extract. The number of eye-stalks represented in the extract injected into each species is stated in the text. All controls received a corresponding amount of sea water, except *Rana* which received 0.7 per cent NaCl solution. All controls gave no response.

It is apparent from these results that the action of the crustacean eye hormone on fish melanophores is to bring about contraction. The case of *Clupea* is at first sight an exception, but we cannot yet regard it definitely so because of lack of accurate information as to the normal processes of pigment cell change involved in adaptation to backgrounds. All other reactions were clear-cut enough to be regarded as melanophore contraction due to the injection of the eye hormone. The response of *Rana clamitans* tadpoles also is confirmatory of previous work.

It had long been supposed that color changes in fishes were due entirely to the influence of the nervous system acting directly upon the chromatophores and that all observed chromatic phenomena were accountable for on that basis, by the function of the central, peripheral, and sympathetic nervous systems. This view was the more firmly held since the innervation of fish chromatophores had been demonstrated anatomically by Ballowitz in 1893. Melanophores of fishes were later found to respond to the secretions of the adrenal and pituitary glands, but the latter were not regarded as essentially necessary for normal pigmentary changes in fishes. In amphibians, on the other hand, it appeared that endocrine secretions controlled pigmentary changes in very large part, if not entirely; while among the reptiles both nervous and endocrine factors were believed to be the governing factors. With the establishment of the importance of humoral substances in amphibian color changes, reinvestigation of the situation in fishes revealed that while the central and sympathetic nervous systems undoubtedly play a large part in chromatophore reactions, all observed phenomena are by no means accounted for entirely on that basis. The evidence for this view has recently been reviewed by Parker (1932). The results here described lend weight to the view that humoral factors are involved in producing color changes in fishes, and that the response to a humoral substance from an invertebrate suggests a more general susceptibility to internal secretions than was first indicated by the reaction to their own or other vertebrate endocrines.

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SUPRAVITAL STUDIES ON THE COLORED CORPUSCLES OF SEVERAL MARINE INVERTEBRATES

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One of the most distinctive cytological features of the vertebrate erythrocyte is the characteristic reaction of its cytoplasm on exposure to appropriate concentrations of such vital dyes as neutral red and brilliant cresyl blue. As a result of this reaction the basophilic substance of the red blood cell is precipitated and aggregated to produce the so-called patterns of reticulation. The reticulation reaction occurs only in the erythrocytic series and seems to be definitely associated with the synthesis of the respiratory pigment, hemoglobin. The basophilic substance reaches a maximum in the early stages of hemoglobin formation and, as the concentration of hemoglobin within the differentiating erythrocyte increases, the basophilic or reticular material decreases. In the mature cells of the mammals this substance entirely disappears, while in other vertebrates it persists to varying degrees depending on the relative degree of differentiation attained by the erythrocytes.

In several of the invertebrates also, respiratory pigments, either hemoglobin or hemerythrin, are found within special cells of the circulating blood or body fluids. It seemed of interest, accordingly, to study the reaction of the invertebrate colored corpuscles to vital dyes in order to ascertain whether the reticulation reaction was specific for vertebrate red blood cells or was characteristic also of similar cells of invertebrates.

In this study four different species were utilized: *Phascolosoma gouldi* (Pourtalès), a gephyrean worm; *Glycera dibranchiata* Ehlers, a polychæte worm; *Thyone briareus* (Lesueur), a holothurian; and *Arca transversa* Say, a mussel. The corpuscles of *Phascolosoma* contain hemerythrin while those of the three others contain hemoglobin. Supravital preparations were made by the dry dye-film method (Dawson, 1932), using neutral red alone, neutral red in combination with Janus green B, and brilliant cresyl blue. Smears stained by Wright's method were also successfully prepared, but the initial application of the undiluted dye was prolonged to three or four minutes followed by a brief differentiation in the diluted dye.

PHASCOLOSOMA GOULDI

In this form the body cavity is relatively voluminous and contains a large amount of corpusculated fluid. This was readily drawn off from unanesthetized animals by means of a small hypodermic needle. The fluid is a pale rose color, due to the presence of numerous hemerythrin-containing cells suspended in the colorless plasma. The body fluid also contains numerous ova or spermatozoa. Since the activity of the spermatozoa makes supravital observations almost impossible, females were used in this study.

The corpuscles usually appear circular in outline and vary widely in diameter. When viewed on edge (Figs. 7, 8) they are seen to be relatively thick, slightly biconvex discs. The variation in size could not be correlated definitely with the degree of maturity of the cells since very large cells were observed with low concentrations of hemerythrin and vice versa. The variations in intensity of the pigmentation of the cells may also be due in part to varying degrees of reduction of the hemerythrin.

Owing to the density of the hemerythrin, distributed uniformly throughout the cell, the nucleus can rarely be distinguished in fresh, unstained preparations. Numerous fine granules, either colorless or light yellow, are seen in the interior of the cell. They may exhibit Brownian movement. Other large, colorless, more refractile vacuoles or globules may also be recognized. In dry-fixed smears stained by Wright's method the granules take a basophilic tint while the large vacuoles appear distorted and empty. Both bodies tend to be grouped about the small nucleus which occupies an eccentric position in the cell (Figs. 1 and 2). When stained supravitally with neutral red, the small granules are deep red while the vacuoles remain uncolored (Fig. 3). When Janus green B is added numerous granular mitochondria are seen distributed irregularly throughout the cytoplasm (Fig. 4).

The most striking staining patterns are produced with brilliant cresyl blue applied supravitally in concentrations high enough to stain the nucleus. The latter then appears light blue, the granules dark blue, and the vacuoles light pink. In addition a fine, flocculent, violet-colored material appears in the cytoplasm (Figs. 5 and 6). This eventually becomes aggregated into more or less definite strands or filaments resembling closely the patterns of reticulation which can be produced by the same staining method in the erythrocytes of vertebrates. The pink reaction of the large vacuoles with brilliant cresyl blue suggests that they are fat vacuoles. No induction or neo-formation of bodies stainable with neutral red was obtained even after long exposure to vital dyes.

GLYCERA DIBRANCHIATA

Glycera lacks a definite, organized circulatory apparatus and the hemolymph with the suspended corpuscles is readily obtained by aspiration directly from the body cavity. The corpuscles are relatively large and have the form of thin, slightly biconcave discs (Fig. 12). They are usually circular, occasionally oval, in outline. In fresh, unstained preparations the hemoglobin is seen to be uniformly distributed throughout the cell, usually in sufficiently high concentrations to completely mask the nucleus. Three different types of cytoplasmic inclusions can be identified without staining: several small yellow or orange granules, one or two large highly refractile globules, and numerous small vacuoles.

In dry-fixed smears stained by Wright's method a small, spherical, chromatic nucleus, usually eccentrically placed, may be seen. Occasional cells are bi-nucleated. The yellow and orange granules are basic in reaction and appear as black, brown, or blue bodies. The large refractile globules and small vacuoles are clear and uncolored.

After supravital staining with neutral red the colored granules appear deep red or brown. The large refractile bodies are light yellow and the small vacuoles are unstained (Fig. 9). With brilliant cresyl blue the colored granules are blue-black and the large globules pink. In addition to these inclusions recognizable in fresh cells, brilliant cresyl blue also reacts to produce two additional inclusions. Clusters of reddish-brown, needle-like crystals appear first; later a diffuse violet flocculation, which eventually changes into an irregular filamentous pattern, is also discernible (Fig. 11). The nature of the induced crystals was not determined. They may represent crystals of hemoglobin or hematin. The reddish-brown color is probably due to the addition of some component of the metachromatic brilliant cresyl blue to the original color of the crystals. The filamentous structures appear to be homologous with the reticulation patterns of vertebrate erythrocytes. Janus green B demonstrates numerous small, granular bodies scattered throughout the cell and these have been identified as mitochondria (Fig. 10). The large refractile globules are probably fat, but the nature and significance of the numerous small vacuoles within the corpuscles is problematic.

THYONE BRIAREUS

Hemolymph containing suspended corpuscles can be obtained directly from the perivisceral fluid of living animals by simple aspiration with a hypodermic needle. However, the corpuscles were found to be more numerous in the Polian vesicles (Van der Heyde, 1922; Kawamoto, 1927) and in most instances the cells studied were obtained from this source. The neck of the vesicle was lightly grasped by a pair of flat forceps, the vesicle clipped free with scissors and transferred to the slide where it was ruptured by pricking with a needle.

The corpuscles are circular in outline and have the form of thin, slightly biconvex discs. In fresh, unstained cells the most conspicuous cytoplasmic inclusions are bright, yellowish-brown granules. They are relatively large and are found to vary in number. Occasionally they are entirely absent but usually one, two, or three, rarely four, are found. The hemoglobin is uniformly distributed throughout the cytoplasm with the exception of a narrow, peripheral zone, and the nucleus is usually invisible. The peripheral zone lacking hemoglobin appears alveolar in optical sections of the corpuscle (Fig. 16); with high focus the surface of the cell appears pebbled, being studded with small, closely packed vacuoles (Fig. 17).

After Wright's stain on dry-fixed smears a small, spherical nucleus is visible. It usually lies away from the center of the cell (Figs. 13 and 14). The colored granules are either unstained or appear brownish-black. The latter appearance is interpreted as evidence of a slight basophilia. Small colored spherules, lacking nuclei and staining with eosin, also occur rather frequently. They may or may not contain a colored granule (Fig. 15). They are probably identical with the minute corpuscles of Kawamoto (1927), who regards them as being devoid of hemoglobin. The cytoplasm of the corpuscles stains intensely with eosin except for a narrow, peripheral zone. This represents the alveolar border seen in fresh cells; in dry-fixed cells the alveolar feature was not evident. A similar unstained surface zone was described by Kawamoto (1927) for the corpuscles of *Caudina chilensis*.

The colored cytoplasmic granules stain intensely with both neutral red and brilliant cresyl blue and in old preparations show active Brownian movement. Mitochondria are readily demonstrated with Janus green B. They appear granular at first, but as the preparation ages and Brownian activity becomes more marked they are seen to be short rods oriented at right angles to the flattened surfaces of the cell. However, the rod-like nature is apparent only when their normal orientation is disturbed by Brownian movement. As in *Phascolosoma* and *Glycera*, high concentrations of brilliant cresyl blue cause a definite filamentous structure to appear throughout the cytoplasm (Fig. 18). This also has the characteristic reddish-violet tint. No fat globules or other vacuoles were observed in the corpuscles of *Thyone*.

Arca transversa

When the valves of a specimen of *Arca* are separated there is usually a considerable collection of red fluid to be found in the mantle cavity. This is described by Cuénot (1891) as being forced out of the circulation by the contraction of the large sinus of the foot. Hemolymph may also be obtained by breaking through the shell in the region of the umbo and aspirating fluid directly from the cardiac region.

The corpuscles of *Arca* are relatively small and either circular or oval in outline. When viewed on edge they are seen to be extremely thin, slightly biconvex discs. The hemoglobin is uniformly distributed throughout the cytoplasm and the nucleus can rarely be distinguished in fresh cells. The only visible cellular inclusions in unstained preparations are numerous yellow-brown granules of irregular size and shape which exhibit varying degrees of Brownian activity.

After Wright's stain a small, spherical nucleus, generally eccentric, is visible (Figs. 19 and 20). The colored granules are conspicuous on the eosinophilic background and usually do not react appreciably with any component of Wright's stain. In some preparations they appear brownish-black, probably indicative of a slight degree of basophilia. The granules stain deeply with supravital applications of neutral red. They also appear to lose their irregular outline and become spherical on long exposure to the dye. After assuming the spherical form they may increase in size, but no new staining loci appear within the cell (Fig. 21). It is not entirely clear whether the granules actually change form or are completely obscured by a densely stained vacuole which develops about them. In old preparations in which the corpuscles have lost much of their hemoglobin the stainable bodies are less densely colored and a small refractile center can be distinguished within them, suggesting that the material of the granule has partially dissolved in the fluid of the enveloping vacuole.

Janus green B brings out a variable number of small, perinuclear bodies which appear either as granules or short rods. These are interpreted as mitochondria (Fig. 22). With brilliant cresyl blue the irregular granules take a deep blue stain and round up as after treatment with neutral red. In the early stages of staining a fine, granular material is seen scattered throughout the cell (Fig. 23) but later this is aggregated into rather indefinite, irregular filaments (Fig. 24).

The corpuscles of *Arca* are quite sensitive to changes in the osmotic pressure of the medium in which they are suspended (Sato, 1931), and typical crenation, of the thorn-apple type encountered in vertebrate erythrocytes, is readily produced (Fig. 25). Crenated corpuscles frequently rupture and one or several balloon-like formations may appear on their surfaces (Figs. 26 and 27). So far as could be determined, only the more fluid contents of the corpuscle, including the hemoglobin, enter the bud-like protuberances, the colored granules and nuclei al-

PLATE 1

All figures were outlined at the same magnification (1200 diameters) by means of a camera lucida. Cells were selected to show the range in size within each species.

Phascolosoma gouldi:

1 and 2. Corpuscles from a dry-fixed film stained by Wright's method showing eccentric nucleus, basophilic cytoplasmic bodies, and unstained globules or vacuoles.

3. Fresh corpuscle stained supravitally with neutral red. The basophilic bodies (black) stain intensely while the clear globules are uncolored.

4. Fresh corpuscle stained supravitally with Janus green B showing mitochondria.

5 and 6. Fresh corpuscles stained supravitally with brilliant cresyl blue showing the basophilic bodies (black) stained deep blue, the clear globules stained pink, and the reticulation filaments stained violet.

7 and 8. Fresh corpuscles seen on edge.

Glycera dibranchiata:

9. Fresh corpuscle stained supravitally with neutral red. Bodies (black) which were yellow or orange in unstained cells were colored deep red. Large clear refractile globules (gray) stained yellow. Numerous small clear vacuoles were uncolored.

10. Fresh corpuscle stained supravitally with Janus green B showing only mitochondria.

11. Fresh cell stained supravitally with brilliant cresyl blue. The nucleus was colored light blue. The small bodies (black) were deep blue, the two larger globules (gray) pink, and the reticulation filaments violet. Violet needle-like crystals also appear after treatment with brilliant cresyl blue.

12. Fresh corpuscle seen on edge.

Thyone briareus:

13, 14, and 15. Corpuscles from a dry-fixed film stained by Wright's method. Refractile bodies, colorless, yellow or brown in fresh cells, give a basic reaction. Fig. 15 shows a small, non-nucleated corpuscle.

16. Fresh corpuscle stained supravitally with neutral red and Janus green B showing the refractile bodies (black) which were deep red and the mitochondria (gray). This corpuscle is shown in optical section to demonstrate the vacuolated or alveolar periphery.

17. Fresh corpuscle at high focus to show the pebbled or vacuolated appearance of the surface of the cell.

18. Fresh corpuscle stained supravitally with brilliant cresyl blue: nucleus, light blue; nucleolus, deep blue; refractile bodies, purple or deep blue, and the reticulation filaments, violet.

Arca transversa:

19 and 20. Corpuscles from a dry-fixed film stained by Wright's method. The cells contain irregular, yellow or brown granules which give a slightly basic reaction.

21. Fresh corpuscle stained supravitally with neutral red. The refractile bodies stain intensely with neutral red and become spherical in form.

22. Fresh corpuscle stained with Janus green B showing only the mitochondria.

23. Fresh corpuscle stained with brilliant cresyl blue showing the early flocculent aggregations induced by the action of the dye.

24. Fresh corpuscle stained with brilliant cresyl blue showing the later filamentous form of the reticulation substance. The refractile granules stain deep blue and round up as with neutral red.

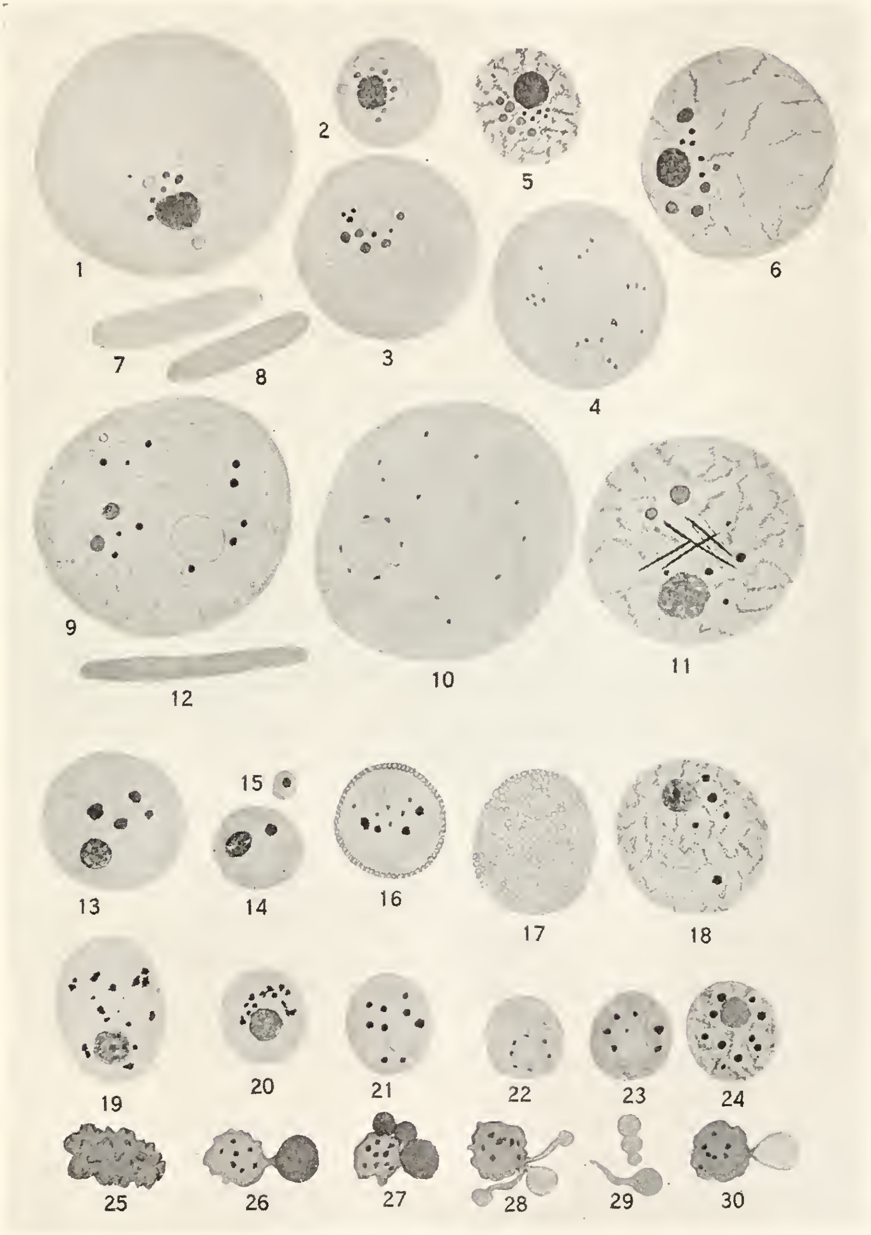
25. Crenated corpuscle from a fresh preparation.

26. Rupture of a crenated corpuscle. The nucleus and refractile granules remain in the body of the cell while the hemoglobin enters the balloon-like extension.

27. Same as above but with three such extensions.

28, 29, and 30. Similar cells after 18 hours in a sealed preparation. Most of the hemoglobin has been lost, revealing the delicate investing membrane of the cellular extensions.

PLATE I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ways remaining in the main portion of the cell. After supravital staining with brilliant cresyl blue the reticulation reaction is obtained only in the buds. In preparations allowed to stand overnight the hemoglobin is usually lost from the buds and a delicate, investing membrane is then visible (Fig. 30). The buds may also change in form, appearing as small knobs attached to the main portion of the cell by slender stalks (Fig. 28). They then exhibit Brownian activity and in many instances become separated from the corpuscles (Fig. 29) to float freely in the hemolymph.

Discussion

It is frequently stated in textbooks that among the invertebrates there are no cells which are exactly comparable to the erythrocytes of the vertebrates. The invertebrate colored corpuscles are described as nucleated, ameboid cells resembling white corpuscles, each cell containing a small globule of pigment in its cytoplasm. Such statements are in direct opposition to all published descriptions of the invertebrate cells which carry respiratory pigments (Cuénot, 1891; Romieu, 1923, and others). These cells are usually nucleated, but are not ameboid, and the respiratory pigment is uniformly distributed throughout the cytoplasm. They are invested by a definite membrane, which appears to be elastic since the corpuscles readily recover typical form after distortion. They hemolyse in the same manner as vertebrate erythrocytes and ordinarily respond similarly to changes in osmotic pressure, exhibiting characteristic swelling and crenation.

In the four species examined at Woods Hole all the corpuscles are circular or oval in outline and have a disc-like form, but the relative thickness of the cells differs in each species. The size of the cells is more variable within the species than in the vertebrates, but the dimensional proportions of the cells of each species are nearly constant. Spherical corpuscles have been described in *Thallasema mellita* by Abbott (1913) and in *Urechis caupo* by Baumberger and Michaelis (1931).

Non-nucleated corpuscles have been reported for only one species of worm, *Magelona papillicornis*. In this form the respiratory pigment is hemerythrin. The early observations of McIntosh (1878) and Benham (1896) regarding the uniform absence of the nucleus have been confirmed by the more recent studies of Romieu (1923). Small, non-nucleated spherules were encountered in the hemolymph of *Thyone* in both fresh preparations and stained smears. Many contained characteristic, colored, granular inclusions and the plastids appeared to be similar in constitution to the larger nucleated elements. No evidence regarding their origin was obtained. Presumably they arise by a process of budding.

The cytoplasmic inclusions of the invertebrate corpuscles are of several kinds: clear vacuoles of undetermined nature, fat globules, and granules. The granular inclusions appear to be characteristic of almost all invertebrate corpuscles whether the respiratory pigment be hemoglobin or hemerythrin. They react readily with vital dyes and may exhibit varying degrees of basophilia. In fresh unstained cells they are readily recognized by their high refractive index and are usually yellow or brown in color. Their nature was not determined. In the literature they are frequently referred to as excretion granules. In *Thallasema*, Abbott (1913) obtained a positive test for iron in these granules, and Baumberger and Michaelis (1931) identified the brown granular pigment in the corpuscle of *Urechis* as hematin.

Vital dyes have not been used extensively in the study of the invertebrate corpuscles, and in most instances when they have been applied the concentration was only sufficient to stain the preformed granular inclusions. However, Romieu (1923) found that in *Notomastus* vital staining with high concentrations of dye caused the appearance of a coarse-meshed network whose branches tended to radiate from the nucleus to the periphery of the cell. He also obtained a somewhat similar pattern in the corpuscles of *Glycera tesselata* after hemolysis and suggested that it might correspond to the substantia granulo-filamentosa (reticulation substance) of the vertebrate erythrocyte.

The present observations show that in the four species studied, patterns resembling the vertebrate reticulation can be obtained by the application of brilliant cresyl blue in suitable concentrations. This staining reaction may be obtained without causing hemolysis but the nucleus is always stained. Similarity of staining reaction and of morphology of the intracellular patterns are admittedly inadequate criteria to establish the identity of the substances occurring in the invertebrate and vertebrate corpuscles, but the uniformity of the reaction suggests that in the elaboration of an intracellular respiratory pigment, whether hemoglobin or hemerythrin, the synthetic processes are fundamentally alike.

SUMMARY

Supravital studies of the colored corpuscles of the hemolymph or body fluid of four species of invertebrates, *Phascolosoma gouldi, Glycera dibranchiata, Thyone briareus,* and *Arca transversa* are recorded. The corpuscles of *Phascolosoma* contain hemerythrin; the others, hemoglobin.

All the corpuscles are disc-like in form and the respiratory pigment is uniformly distributed throughout the cell.

The corpuscles are usually nucleated, but in *Thyone* a variable number of small, non-nucleated spherules were noted.

Mitochondria were present in all cells. Other cytoplasmic inclusions consisted of vacuoles of undetermined nature, fat globules and refractile granules, either colorless, yellow or brown. The granules react readily with vital dyes and are slightly basophilic. Their composition was not determined but it is suggested that they are derivatives of the respiratory pigment.

Rather extensive patterns of reticulation, resembling those of vertebrate erythrocytes, appear in the corpuscles of all four species on exposure to suitable concentrations of brilliant cresyl blue.

The corpuscles of *Thyone* are characterized by a peculiar alveolar zone immediately beneath the cell membrane.

The corpuscles of *Arca* frequently rupture when crenated and much of the fluid content, including the hemoglobin, enters the thin-walled, balloon-like extensions found on the surfaces of the cells. The nuclei and specific granules remain in the main body of the cell.

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MATING IN LIMULUS POLYPHEMUS[1]

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Homogamy, the tendency of like to mate with like, has been definitely established for several plants and animals. A general account especially from the plant side has been given by D. F. Jones (1928). Willoughby and Pomerat (1932) have summarized the zoölogical findings. In a study by H. E. Jones (1929) and one by Schiller (1932), the literature dealing with homogamy in psychic characters in man has been reviewed.

The factors involved in such selection may be simple morphological characteristics depending upon the mechanics of the mating act. The liberation of specific substances preceding mating may also play a rôle by stimulating olfactory receptors of the opposite sex. Complex behaviour, degree and extent of activity, and more specifically preliminary sex behaviour, may also be important in the process of selection. This involved mating behaviour in man is generally referred to as psychic, and is usually described in psychological terminology. Homogamy in man may depend largely upon psychic factors, while activity (as a function of age) may be responsible for the relatively high degree of correlation in toads (Willoughby and Pomerat, 1932). Mechanical adjustment of the structures involved in the mating act may afford the simplest explanation for selective coupling since, a priori, it would seem that successful mating is possible only within certain limits of form which in turn are conditioned by (1) the complexity of the coupling, *i.e.*, the number and character of the points of contact and (2) the degree of mobility of the body to allow for adjustment. To these variables a third might be added—relative activity and physical vigor—since successful mating may require pursuit and capture, display mechanisms, and (at least in the cases where amplexus is involved) that, for a period, the participants be attached to each other.

In a previous paper (Pomerat, 1932) it was pointed out that results obtained from the study of homogamy in invertebrates were not, contrary to previous opinion (Willoughby and Pomerat, 1932), consistent among themselves. Workers in this field have tended to suppose that

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[1] This investigation was aided by a scholarship from the Collecting Net.

cœfficients of correlation for homogamy might be high for organisms having relatively inflexible exo-skeletons. This would seem especially possible, from a mechanical standpoint, for species practicing internal fertilization where at least two points of contact are involved. This, however, was not found to be the case in a study of the Japanese beetle, *Popillia japonica* Newm. (Pomerat, 1932), in which the usual dorsoventral position is taken, with clasping by the male in addition to the genital contact. It was not found possible to isolate the chief mechanical structures upon which amplexus is dependent, unless the structures studied, namely the four tibia and tarsi, actually are critical in the process. The central tendency of the distribution of cœfficients derived from eight different measures was very slightly positive in the Japanese beetle. In view of this unexpected finding it was thought advisable to examine the mating behaviour of other arthropods. The species selected for this study, the horse-shoe crab, *Limulus polyphemus* L., is of particular interest since it represents a form in which copulation involves amplexus but with no intromission, so that there is only one pair of contact points involved in the coupling, *i.e.*, clasping without genital contact. It was conjectured that the removal of the partial restriction imposed by genital contact might result in an increased range of choice and consequent lowered cœfficients. A significant difference in the latter, as between the two types of mate-union, might thus afford some support for the theory of mechanical determination of homogamy. In addition, *Limulus* is the largest invertebrate which has been studied thus far from the standpoint of homogamy. Considering technical difficulties involved in obtaining biometric data, the horse-shoe crab owing to its large size should form a relatively more reliable source of information than the arthropods heretofore examined.

Materials and Methods

One hundred pairs of the horse-shoe crab, *Limulus polyphemus* L., were taken in the vicinity of Woods Hole, Massachusetts. The largest group (49 pairs) was found along the sandy beaches on either side of Penzance Point. All measurements were taken between June 1 and June 5, 1932. Mated *Limuli* are most often found after sun-down, at high-tide, in shallow water along protected sandy beaches. According to Mr. George Gray of the Marine Biological Laboratory Museum at Woods Hole, the height of mating activity is reached during the full moon late in May or early in June. Kingsley, writing in 1892, stated that he had never found this correlation. Unfortunately the writer was unable to confirm either view.

By means of modified pincers of the second pair of feet the male grasps the hinder half of the abdomen of the female and trails along in shallow water. Locomotion of mated pairs being relatively slow makes the collection of animals easy.

All measurements were taken in inches to the nearest sixteenth. These data were later converted entirely to sixteenths. This system was one of expediency, since at the time of collection it was impossible to procure a steel tape marked in metric measure for use on curved structures.

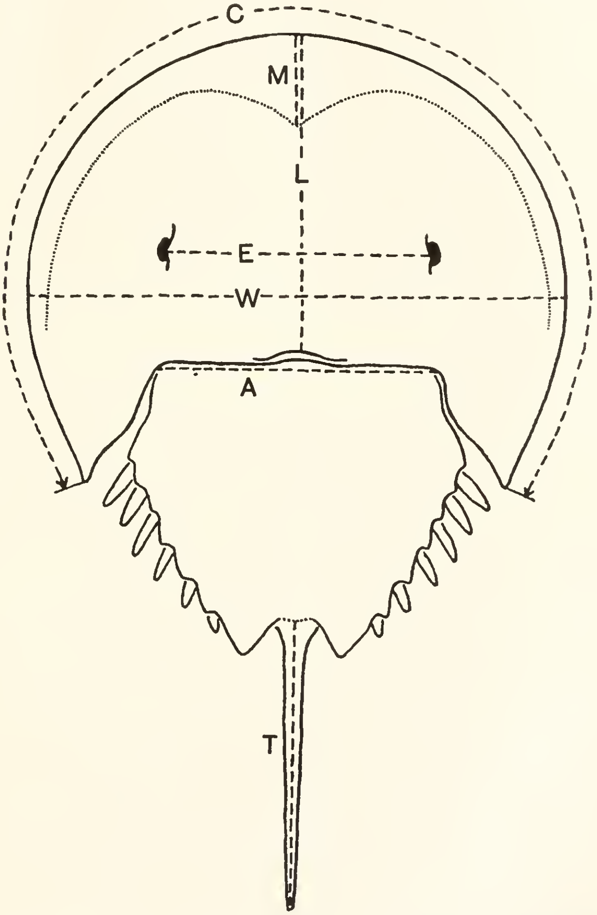

FIG. 1. Diagram of the dorsal aspect of *Limulus polyphemus* illustrating measurements taken.

The outline of the animal is shown by unbroken lines (eyes in solid black); dotted lines indicate structures of the ventral surface; interrupted bars show the course of the seven measurements.

Table I

W	90	95	100	105	110	115	120	125	130	135	140	145	150	155
m	1			1	6	9	26	21	16	3	12	3	1	
f									1		3	6	5	5

	160	165	170	175	180	185	190	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m								99	125.1	9.5	7.6		
f	26	13	11	17	7	1	4	99	164.6	12.0	7.3	25.8	1.6

L	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135
m	1		1	7	25	23	20	19	3	1					
f									1	3	4	16	15	18	20

	140	145	150	155	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m					100	131.2	7.5	5.7		
f	12	8	1	1	100	89.9	9.5	5.0	34.1	1.6

M	20	22	24	26	28	30	32	34	36	38	40	42	44	N	M	σ
m	1	2	17	32	27	15	6							100	27.02	2.4
f						2	2	6	29	20	27	7	7	100	38.14	3.0

	V	d_M/σ_d	d_σ/σ_d
m	8.8		
f	7.8	34.1	1.6

C	200	205	210	215	220	225	230	235	240	245	250	255	260	265
m	2	8	14	7			1							1
f														

	270	275	280	285	290	295	300	305	310	315	320	325	330	335
m		2	3	2	6	8	5				6	7	4	7
f													1	

	340	345	350	355	360	365	370	375	380	385	390	395	400	405
m	3	2	4	5	1	1	1							
f			2	2	1	1	1	2	4	4	9	3	4	3

	410	415	420	425	430	435	440	445	450	455	460	465	470	475
m														
f	13	7	1	9	8	5	5	5	3	2	2		1	1

	480	485	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m			100	283.8	55.0	12.3		
f		1	100	412.85	29.0	7.0	20.9	4.2

E	55	60	65	70	75	80	85	90	95	100	105	110	115	120	125
m	1	1	15	14	33	26	10								
f						1	3	8	21	17	24	18	7		1

	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m	100	74.75	6.5	8.7		
f	100	101.8	8.0	7.8	26.2	1.4

T	65	70	75	80	85	90	95	100	105	110	115	120	125	130	135
m	1		2	1	1	4		9	9	10	13	7	11	6	3
f								2		3	5	3	1	4	8

	140	145	150	155	160	165	170	175	180	185	190	195	200	N
m	4	1		1										83
f	13	10	8	6	7	7	1	3			1		1	83

	M	σ	V	d_M/σ_d	d_σ/σ_d
m	113.75	16	14.0		
f	144.00	19	13.2	11.0	1.1

TABLE I (*Continued*)

A	50	55	60	65	70	75	80	85	90	95	100	105	110	115	120	N
m	1	2	11	39	30	12	5									100
f				1		3	10	16	20	31	13	3	2		1	100

	M	σ	V	d_M/σ_d	d_σ/σ_d
m	67.55	5.5	8.1		
f	91.50	8.0	8.7	24.7	2.5

Seven measurements were taken and recorded in the field. These were grouped and designated (see Fig. 1) as follows:

I. Circumferential Measure.

C—curved distance around cephalothorax from one posterior marginal spine to the other.

II. Median Sagittal Measures.

M—length of median widening of the rim of ventral carapace;

L—length of cephalothorax along its curvature;

T—telson length, ventral aspect measured from the ridge distal to muscular attachment of the base to the tip.

III. Width Measurements.

E—curved distance between the median edge of the compound eyes;

W—greatest width of the cephalothorax taken straight across the ventral side;

A—width of abdomen at the cephalothoracic junction.

The distribution of direct measures is given in Table I.

It is evident that the females are significantly larger and cover a wider range of sizes than the males. It should be noted that the scatter of measurements for the character *C* (circumference of the cephalothorax) in the male shows a trimodality with peaks in the vicinity of 210, 290, and 335. Of the following cœfficients, those in which this measure is involved are slightly incomparable with the others for this reason.

One hundred and four Pearson product moment correlations were obtained from 1400 measurements taken on 100 pairs of mated *Limuli*. These will be considered in the following order: (1) direct and cross-correlation, (2) self correlations, (3) quotient correlations.[2]

Direct and Cross Correlations

The results of correlations of these measurements are given in Table II.

Direct correlations range from — 0.005 to 0.151. The lowest value obtained (— 0.005) existed between the abdominal width of mated pairs. This was the lowest value found for the entire series. The highest direct correlation value (0.151) was found for the cephalothoracic width of mates.

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[2] It is a pleasure to acknowledge indispensable assistance from Miss Edith Fishman, and from Mr. James Sell.

Cross correlation values range from — 0.01 to 0.246; the former value existing between the median width of the rim of the ventral carapace of the female and the male cephalothoracic circumference, while the latter, the highest value for both direct and cross correlations, was found for the relation between the telson length of the female and the cephalothoracic width of the male. It must be noted, however, that the telson length cannot be absolutely relied upon since it was frequently found that the telson was eroded to a varying degree, and in a few cases it was broken. These accidents arise from the horse-shoe crab's habit of burrowing in sandy and muddy shores. The crab first arches its body by bending it at the joint between the cephalothorax and the abdomen, draws the sixth pair of legs forward and then extends the body, pushing with the legs against the firm hold of the telson. In view of the unreliability of the measurement, *T*, it is interesting to note that the next highest measurement (0.202) is found between the cephalothoracic circumference of the female and the median widening of the rim of the ventral carapace in the male.

Table II

Direct and Cross Correlations Obtained for Seven Characters Measured on 100 Mated Pairs of Limulus polyphemus L.

		Males							
		W	L	M	C	E	T	A	
Females	W	0.151	0.104	0.118	0.036	0.083	0.044	0.087	
	L	0.107	0.147	0.118	0.090	0.101	0.073	0.079	
	M	0.084	0.081	0.111	−0.010	0.083	0.030	0.090	
	C	0.136	0.156	0.202	0.103	0.128	0.042	0.083	$M_r = 0.10$
	E	0.161	0.121	0.182	0.044	0.101	0.020	0.062	
	T	0.246	0.153	0.150	0.004	0.120	0.081	0.080	
	A	0.076	0.022	0.150	0.020	0.023	0.001	−0.005	

The abdominal width measure *A* in the female appears to influence low correlations most frequently; five cases with values of less than 0.025 occurring in the series. The greatest cephalothoracic width *W* and the median sagittal width of the ventral carapace *M* in the male seem to influence high correlations; four values of more than 0.130 being found for each in the series. The mean value for 49 direct and cross correlations is approximately 0.100. With a probable error of ± 0.06 it is quite possible that the values obtained are chance results. Measurements on at least 500 individuals would be necessary to lower the probable error enough to obtain fixed values. The correlation values obtained, however, strongly suggest that homogamy as regards size in

Limulus polyphemus L. is practically non-existent. If a slight correlation should turn out to be real and not merely due to sampling, it might conceivably be explained on the basis of the slight limitations imposed by the adjustment of the male clasping claw to the female carapace.

Self Correlations

This designation is used for correlations between different measures of the same individual. See Table III.

The means of the 42 self correlations possible with seven measures were 0.65 for the males and 0.78 for the females. The highest coefficients (0.97 for both sexes) were those expressing the relationship between interocular width and cephalothoracic length; the lowest for the male (0.23) was between the cephalothoracic circumference and the abdominal width, and for the female (0.51) that between the telson length and the greatest distance across the rim of the ventral carapace. The influence of the circumference of the cephalothorax measure on other characters seems to operate in opposite directions in the male and in the female. The cephalothoracic measurement of the female, for the most part, combines with every other measurement to give high correlations, whereas the cephalothoracic measurement of the male never does; for example, self correlation *C–A*, where the male gives 0.23 and the female 0.95. It is significant that the largest measure taken, the circumference of the carapace, is related with every other characteristic studied to give high self correlation (consistency of proportion) in the female, while in the male this tendency is relatively negligible.

Table III

Coefficients for Self Correlations

	E–L	*L–A*	*L–W*	*A–W*	*E–A*	*E–W*	*N–W*	*M–E*	*M–L*	*M–A*	*T–A*
m	0.97	0.89	0.88	0.85	0.84	0.83	0.77	0.73	0.72	0.70	0.59
f	0.97	0.84	0.88	0.84	0.89	0.85	0.82	0.76	0.82	0.79	0.49

	T–L	*T–E*	*T–W*	*C–W*	*C–L*	*M–C*	*T–M*	*E–C*	*T–C*	*C–A*	Mean
m	0.59	0.57	0.55	0.52	0.51	0.51	0.48	0.45	0.33	0.23	0.65
f	0.56	0.52	0.62	0.94	0.96	0.82	0.51	0.91	0.65	0.95	0.78

In the seven characteristics studied the female was found to show the greater range in size, the greater variability, and a more marked consistency of form. Low self correlations between telson length and all other measures in both male and female further serve to indicate that the telson measure has a prominent chance error in it. If self correlation values are eliminated when they contain the telson measure the means are raised noticeably; for the male the M_r becomes 0.69 and for the female 0.87. This greater variability and higher self correlation in the female has also been found for the toad by Willoughby and Pomerat (1932) and for the Japanese beetle by Pomerat (1932).

Table IV

C/W	150	155	160	165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	240	245	250	255	260	265	270	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m	2	1	7	14	7	1	1								2		1	6	16	12	16	5	6	1	1	99	218.85	39	13.2		
f																	1	4	19	28	31	14	1		1	99	246.85	6	2.7	7.1	8.4

L/W	64	66	68	70	72	74	76	78	80	82	84	86	88	90	92	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m	2	1	5	26	29	16	14	5								98	72.24	2.8	3.8		
f					1	11	25	19	25	9	6			1	1	98	78.48	3.2	4.0	14.8	0.95

E/W	52	54	56	58	60	62	64	66	68	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m	1	3	12	29	23	20	10		1	99	59.54	2.8	4.7		
f			2	16	27	25	21	5	3	99	61.50	2.6	4.2	5.1	.52

A/W	46	48	50	52	54	56	58	60	62	64	66	68	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m		3	4	18	39	26	6	2		1			99	56.28	2.4	4.2		
f	1		3	12	26	39	13	3		1		1	99	53.30	2.8	5.2	8.2	1.1

T/W	51	54	57	60	63	66	69	72	75	78	81	84	87	90	93	96	99	102	105	108	111	N	M	σ	V	d_M/σ_d	d_σ/σ_d
m	1			1	1	2	1		5	2	5	6	7	12	10	6	14	3	3	1	2	82	89.61	11.1	12.3		
f						2	1	2	8	10	7	8	10	10	10	2	8	3	1			82	86.10	9.0	10.4	2.3	1.4

Quotient Correlations

Quotients obtained by dividing one character by another indicating other sex differences are as given in Table IV.

Quotient or morphological indices were correlated directly in fourteen cases. See Table V.

TABLE V

Quotient Correlations (Morphological Indices)

M/C	0.16	E/A	0.05	M/E	−0.07
C/W	0.14	E/W	0.03	T/W	−0.08
L/W	0.11	L/E	0.01	M/A	−0.11
L/M	0.11	T/L	−0.01	C/A	−0.225
C/E	0.08	A/W	−0.01	M_r	0.086

Quotient values were treated to obtain correlations for form. The consistently low values obtained gave a mean value of 0.086, which would suggest that if there were a real, though slight, homogamy in *Limulus,* this would depend upon size rather than form.

DISCUSSION

A biometric study of one hundred mated pairs of the horse-shoe crab, *Limulus polyphemus* L., was made to test the theory of mechanical determination of homogamy.

TABLE VI

Correlation Coefficients for Homogamy in Invertebrates (Averages)

Species	Character	r	Author
Paramecium	Length	0.61	Pearl (1907)
	Breadth	0.30	
	Index (Breadth/Length)	0.43	
	Length (wild cultures)	0.38	Jennings (1911)
	“ (two species)	0.94	
	“ (pure races)	0.23	
	Breadth (wild cultures)	0.33	
Chilodon uncinatus	Length	0.40	Enriques (1908)
Chromodoris zebra	Mantle length	0.52	Crozier (1918)
	Total length	0.61	
Gammarus locusta	Length	0.91	Crozier and Snyder (1923)
	Length	0.80	Crozier (1918)
Dikerogammarus fasciatus	Length	0.69	Crozier and Snyder (1923)
Popillia japonica	Length	0.19	Pomerat (1932)
	Thoracic Width	0.18	

Coefficients of correlation have been found to be high for species in which precise apposition of contact points is necessary for copulation, as indicated by Table VI.

Conjugation in the protozoans, *Paramecium* and *Chilodon;* hermaphroditism in *Chromodoris;* and the complexity of mating in gammarids, as described by Holmes (1903), suggest that it may be possible to account for the high correlations obtained on the basis that the mechanical precision involved does not permit coupling of individuals of widely different sizes.

The findings for the Japanese beetle, *Popillia japonica*, do not tend to uphold this view, unless it can be considered that a greater degree of adjustment of contact points is possible than might a priori be expected.

The findings in *Limulus polyphemus* are in keeping with the mechanical theory, on the basis that only one point of contact is involved. The range of choice is, therefore, markedly increased and the coefficient of homogamy proportionately lowered.

There is some evidence then that mating in certain invertebrates rests at least in part upon a mechanical process.

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PHENOMENON OF REGENERATION IN EVERTED HYDRA

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Introduction and Historical

The first worker to observe that a hydra turned inside out might regenerate was the Swiss priest, Alexander Trembley (1744). Trembley maintained that the ectoderm, which in the turned animal lines the enteric cavity, came to take over the functions of the endoderm; while the endoderm, which was outside, assumed the functions of the ectoderm. Nussbaum (1887, 1891) repeated Trembley's experiment, but came to the conclusion that, instead of reversal of function, migration of the layers took place. He described sliding of the ectoderm over the endoderm, beginning at the mouth and a pore in the base, and continuing until the entire ectoderm had reached its outside position. Ischikawa (1890) undertook the same problem and obtained the same results as Nussbaum, but he explained them much more plausibly. Both Nussbaum and Ischikawa criticized Trembley for his manner of proving that the everted animals remained everted and did not revert themselves. The present paper deals with the changes occurring in ectoderm and endoderm after the position of these two layers has been reversed by turning. The actual explanation of the regeneration phenomenon is quite different from any previously offered.

Materials and Methods

For this work specimens of three common species, *Hydra viridissima* Pallas (=*viridis* L.), *H. vulgaris* Pallas (=*grisea* L.) and *H. oligactis* Pallas (=*fusca* L.), have been used, each in the manner in which it was best suited. The first proved to be best for studies on the living material; the latter two were better for the study of fixed and stained material.

The method of turning was very simple. A hydra in a small dish with a few drops of water under a binocular microscope was stimulated to contract by careful prodding with a needle. When it had fully

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[1] The writer feels greatly indebted to Dr. S. H. Derickson and Dr. V. Earl Light of Lebanon Valley College, and to Dr. Elery R. Becker of Iowa State College for guidance and criticism concerning the problem and preparation of the manuscript.

contracted, its anterior end was placed on the bottom of the culture dish, care being taken that its tentacles radiated from the hypostome in the normal manner. Then, very carefully, the basal disk was pushed with a fine needle until it came in contact with the bottom of the dish through the mouth of the hydra. In order to accomplish this, a number of successive movements of the needle, so as to force the mouth open and the body through it down against the glass, was necessary. Then the mouth portion was carefully manipulated with another needle so as to force it to come back up against the first-mentioned needle. The hydra was now turned and rested in an inverted position on the point of one of the needles. It was then very carefully pushed off, and more water added.

Animals to be fixed were allowed to remain in the inverted condition for a predetermined length of time, and then killed with warm corrosive acetic poured onto them in a stream from a fine pipette, beginning at the base. In this way the hydras were killed in an extended condition. This material was stained *in toto* with borax carmine and sectioned as thin as 5 and 10 μ.

Results

In the earlier work previously mentioned it was found that hydras which have been turned fall into three distinct classes according to their subsequent behavior: First, those which are not able to adjust themselves to their new situation, and so almost immediately undergo "depression," or slow contraction accompanied by disintegration, and finally death, unless re-turning and recovery supervene; second, those which attempt to re-turn themselves; and third, those which remain turned and regenerate.

It has been found that, out of a group of 60 hydras taken from their native pool and turned on March 19, 1931, 21 underwent "depression," 18 re-turned themselves, and 21 remained turned and readjusted themselves.

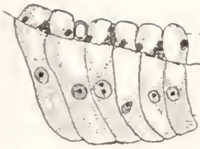
After having observed approximately 300 hydras turn themselves back to their normal condition, the writer can affirm that it took place nearly every time in the manner originally described by Trembley. The everted hydra has its tentacles extending out of its mouth in a group. It will, of course, be realized that hydras in such a condition would generally be contracted to the fullest extent. This results in the mouth being pulled closely around the tentacles which pass out through it. The inside mouth comes to be on the outside, and the hollows of the tentacles, which normally are connected with the enteric cavity, can be seen to open on the outside. In the majority of the specimens some or all of the openings to the outside become closed by the appearance of sphincters within a few minutes after turning. The hydra usually extends the free ends of its tentacles down along the sides of its body and continues to contract and expand them while holding them there. This procedure may pull the mouth of the animal open and cause the edges of the hypostome to be drawn back along the body. By alternate contractions and expansions of the tentacles the mouth is pulled backwards further and further until finally the base itself passes through the mouth. Finally, the contractions and expansions of the muscular elements easily force the basal disk back into its normal position, and then the hydra has recovered its original form.

The recovery of the normal orientation of the two body layers just described is a gross process, crudely comparable to the righting of a glove finger by re-turning it. The third class of turned hydras previously mentioned do not re-turn themselves in the accepted sense of the term, but nevertheless recover a normal organization by rearrangement of the cells constituting ectoderm and endoderm. This regeneration, as will be shown, is effected by migration of ectoderm and endoderm constituents in opposite directions through and beyond the mesoglea.

Sections of one hydra which was fixed two hours after turning showed the ectoderm and endoderm of the trunk in practically their normal positions. Other individuals required up to 8 hours or more to accomplish the same result. One specimen, a brown hydra, showed nematocysts in an exterior position twenty minutes after turning. Another, a green specimen, showed plainly that a considerable number of clear ectoderm cells had penetrated the mesoglea to a position on the outside within 30 minutes. Careful microscopic observations on the living turned *Hydra viridissima* have often disclosed the clear cells in the act of migrating to the outside between the green cells of the endoderm. Such cells become spherical, perhaps on account of freedom from pressure after they have passed through the green cell layer.

The stained sections of *Hydra vulgaris* and *H. oligactis* show the migration of cells very clearly. Figure 1 shows an arc of a cross-section of a specimen killed 10 minutes after the act of turning. Here the ectoderm is definitely on the inside, and no migration has as yet taken place. Figure 2, a section taken from an animal which had been turned 2 hours previous to fixing, shows the migration in progress. No definite mesoglea could be recognized in this animal except in certain isolated portions where the cells had not as yet commenced to move. Ectoderm and endoderm cells are found on both sides of a line drawn continuous with the remaining portions of the mesoglea. The large vacuolated cells are endoderm, while the smaller ectodermal cells are shown mostly without vacuoles.

Plate I



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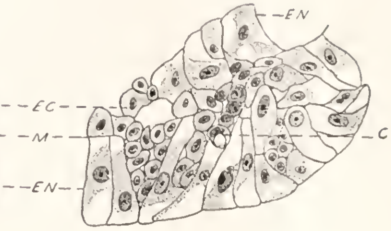

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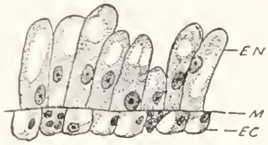

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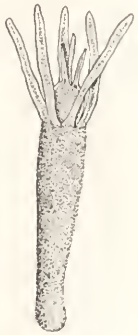

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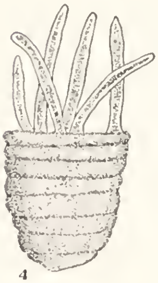

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EXPLANATION OF FIGURES

The upper portion of Figs. 1, 2, and 3 is the interior of the hydra. Here the ectoderm is shown in the internal position. *EN*, endoderm; *EC*, ectoderm; *M*, mesoglea.

Fig. 1. A portion of a cross-section of an everted hydra which was killed 10 minutes after turning.

Fig. 2. Portion of a cross-section of specimen after having remained everted for 2 hours. Some endoderm cells are shown here to have already reached the inside, while some still remain in their everted position. Masses of ectoderm cells are shown migrating through the section. Note the absence of mesoglea in most of the section. *C*, cnidoblast.

Fig. 3. A portion of a cross-section taken from a hydra 24 hours after eversion. Here the cell layers are shown in their normal condition after having completed the migration.

Fig. 4. Hydra immediately after turning, showing the arrangement of tentacles at the anterior end. The mouth of this specimen is not fully contracted.

Fig. 5. Hydra with bud after having been turned. Note the bud coming out of the mouth.

Figure 3 shows the condition of the cells of another specimen 24 hours after turning. The endoderm cells in this section have regained their interior position and proper alignment, and those of the ectoderm have assumed their normal arrangement on the exterior of the mesoglea.

The writer made sections of many specimens which were made too early or too late to show the migration convincingly. The obtaining of favorable material showing the migration at its height is more or less a matter of chance.

In the sponge, Wilson and Penney (1930) have shown that the cells migrate with somewhat amœboid movement, so it does not seem particularly striking that the cells in the hydra can wander. In fact, it has been shown by Hadzi (1909) that nematocysts migrate through the bodies of hydroids. Kepner and Barker (1924) have shown that the nematocysts of hydra can migrate from the enteron to the epidermis of worms of the genus *Microstoma,* which have ingested the hydra as food. In the face of these previous experiments, there is really nothing remarkable concerning the fact of the migration of the cells in the hydra, but they do not explain the method of migration. Personally, the writer feels that the cells migrate by almost imperceptible amœboid movements, although definite pseudopods have not been observed.

The fact of the filtering of ectodermal and endodermal elements through the mesoglea and through each other has been observed clearly in living and sectioned material, and definitely shows the older explanations of the changes occurring during regeneration to be in error. The writer can offer no plausible explanation for the *modus operandi* of the cell migration, which he has shown to occur, beyond the fact that similar phenomena have been observed in other instances.

The writer has no definite explanation for the rearrangements in the anterior end which lead to the reorientation of the tentacles and the hypostome. There appears, however, to be in this region a very complicated migration of cells which is very difficult to follow. The process seems to be initiated by the closing of the pores leading to the hollows of the tentacles, which, of course, are on the outside of the turned hydra.

SUMMARY

1. Hydra may recover and continue to live after having been turned inside out.

2. The reactions to the turning may be classed in three groups: (*a*) "depression" and eventual dissolution; (*b*) re-turning; (*c*) regeneration through cell migration and without any re-turning.

3. Hydras that remain turned regain normality by the migration of the cells constituting the two layers of the body wall.

4. The migrating cells of each layer, individually or in groups, penetrate the mesoglea and filter between the cells of the opposing layer.

5. Hydras regenerated as described usually ingest food after 2 days from the time of turning.

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REACTIONS OF TWO HYDRAS, ONE OF WHICH HAS BEEN INGESTED BY THE OTHER

ANDREW L. PICKENS

It is well known that at times one hydra is swallowed by another, that the victim is retained for some time, and eventually regains its freedom, apparently none the worse for having been ingested. Few, however, have actually observed such an occurrence. One might linger around an aquarium for months, hoping for such an event, only to have his wait climaxed with disappointment. One might even place opposite ends of a small worm into the mouths of neighboring hydras with the hope of seeing the smaller ingested with the worm, only to see the weaker one drawn to the mouth of the other where the remainder of the worm is drawn from one enteron into the next while the losing hydra is permitted to drift away.

Several years ago, it was my pleasure to spend some time at work with hydras at the University of Virginia under the guidance of Dr. W. A. Kepner. Experiments were being made with beef-feeding, and the reactions of these cœlenterates to this unwonted food was indeed marked, and led to the discovery of a method by which one hydra could be made to ingest another, quite at the will of the experimenter.

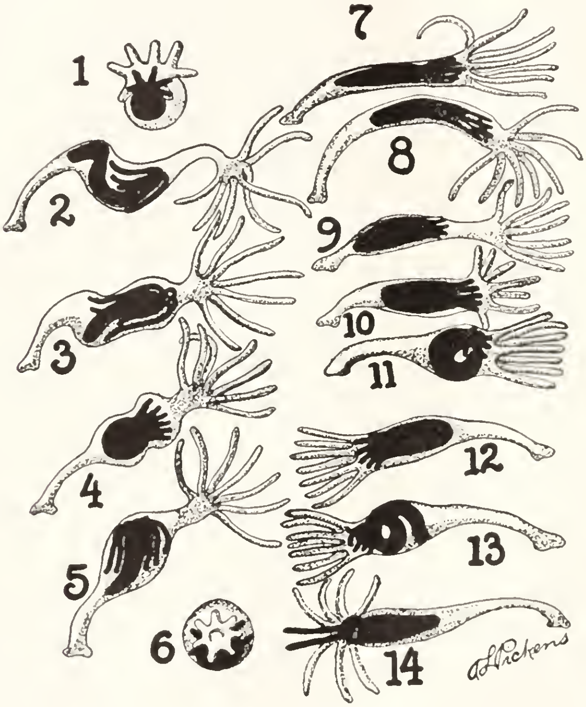
If the point of a dissecting needle is drawn several times through a piece of beef and then presented to the oral end of a hydra, it will receive the point of the instrument into the enteron, closing on the steel tightly with its entire interior length. The animal can then be lifted easily and transported to any part of the vessel. If, in this position, the animal is waved above the mouth of a larger hydra, the latter opens its mouth widely, whereupon the smaller animal may be readily thrust into the gaping enteron below. The reactions of such a pair are shown in the accompanying figures. The smaller was introduced into the gastrovascular cavity of the larger as the hour of 1:00 P.M. approached. The following reactions took place.

The larger animal contracted, assuming an almost globular shape (Fig. 1).

Both animals extended slowly, the contour of the outer fitting itself to that of the inner, the tentacles of which were all reversed. Motion on the part of the inner was followed by another contraction on the part of the outer, whereupon the inner also contracted (Fig. 2).

Another extension on the part of both occurred, and it could be seen that the prisoner's tentacles were being drawn forward. This motion of the interior hydra was again followed by contraction on the part of the outer hydra, immediately followed by a similar motion on the part of its captive (Fig. 3).

The outer animal extended once more. The inner one, as a result of the recent contraction was enabled to bring all its tentacles forward. Again the inner hydra contracted as did the outer (Fig. 4).



FIGS. 1–14. Two hydras, one of which has been ingested by the other. For description, see text.

Once again the prisoner had its tentacles reversed, and as a new extension occurred was seen to have been thrown across the body length of the outer hydra (Fig. 5).

At about 1:15 P.M. the outer hydra swung into a vertical position and again contracted. Observations were hindered by this for some time (Fig. 6).

At 1:30 P.M. the outer hydra was extended and lying prone, while the inner had the tentacles extended to the front. Its position would have facilitated escape through the oral opening of the captor, but the latter contracted once more (Fig. 7).

At 1:45 the outer hydra had assumed the form of an arc in the top of which lay the body of the prisoner. A still longer extension of the outer hydra's body followed (Fig. 8).

The prisoner lost the ground gained, slipping further down and in than ever, touching the very bottom of the captor's enteron (Fig. 9).

An advance movement by the prisoner brought its fore regions into contact with the regions just within the hypostome of the captor (Fig. 10).

The prisoner assumed an almost circular form, which apparently helped it retain its position in the fore-end of the captor (Fig. 11).

At 1:51 the prisoner had straightened out again with the tentacles touching the inner portion of the captor's oral opening (Fig. 12).

The inner hydra, still just within the captor's hypostome, not only threw itself into a circle but actually coiled (Fig. 13).

By 2:28 the prisoner at last extended two of its tentacles through the captor's mouth into the open (Fig. 14). Liberty seemed imminent, when some movement on the part of the outer plunged the prisoner once more into the enteron of its captor. It appeared, however, to maintain its tentacles in the desirable forward-pointing position, and so was enabled to regain its lost ground more readily. At last at 2:51 the captor contracted in a different manner, for this time it evidently reversed the hypostome. Two forward movements were detected on the part of the captive, and it slipped out to freedom so rapidly that I was disappointed at my inability to make more lucid observations of what occurred. It had been a prisoner for approximately two hours.

The species used in this experiment was *Hydra viridissima.*

Additional experiments have shown the method to be practical for laboratory activities. It offers opportunity for tests as to whether or not two hydras repeatedly placed in this unusual relation would be able to sever the union more rapidly after a number of similar experiments had been undergone, as to the reception of macerated hydra flesh compared with living hydras, and other interesting problems.

OBSERVATIONS ON CERCARIÆUM LINTONI MILLER AND NORTHUP AND ITS METACERCARIAL DEVELOPMENT

C. RUTH SHAW

(From the Department of Zoölogy, University of Pittsburgh, the Marine Biological Laboratory, Woods Hole, Mass. and the Department of Biology, Kent State College)

INTRODUCTION

This species was first recorded by Linton (1915), who found it in *Nassa* (=*Illynassa*) *obsoleta* (Say). He gave a brief description of the sporocyst and of the cercariæum, although he did not name the species. His account was confirmed and extended by Miller and Northup (1926), who named the larva in honor of Professor Linton. These authors made a twelve-month survey of the infestation of *N. obsoleta* with larval trematodes and found *Cercariæum lintoni* emerging at all periods of the year, with the greatest infestation in June and December. In a recent paper Africa (1930) described the excretory system of the cercariæum. He gave a figure of the flame cell pattern and a discussion of the mechanism by which the bladder and its accessory sphincter appear to function.

The present report contains the results of work done at the Marine Biological Laboratory during the summer months of 1930 and 1931. The study of marine cercariæ was suggested by Professor H. W. Stunkard, to whom I am indebted for suggestions and advice during the course of the work and in the preparation of this paper. Grateful acknowledgments are made also of assistance from Professor Edwin Linton, Professor P. W. Whiting, and Mr. Raymond M. Cable.

MATERIAL AND METHODS

Great numbers of snails, *Nassa obsoleta*, were obtained from Quisset Harbor and examined by the isolation method for specimens from which *Cercariæum lintoni* were emerging. These infected snails were placed in glass dishes where it was possible to keep them for days and even weeks. A daily change of water sufficed to maintain the snails in good condition and afforded a constant supply of these larvæ for experimental purposes. Morphological details were studied according to the methods discussed by Stunkard (1930). Compressed, unstained, living cercariæ were observed for details of the flame cell pattern. Others, after staining with neutral red, were used in the study of the different glands. Permanent mounts were made also of specimens fixed and stained by various methods.

Experiments were carried on to determine the effects of changes in environmental conditions, and others were performed in an attempt to elucidate the life history and secure the adult form of the species. Certain features of the cercariæ, (1) the presence of a stylet, (2) presence of cephalic, probably penetration glands, (3) failure to encyst on the walls of the container, and (4) great endurance of changes in environmental factors, appeared to be of significance for developmental studies and suggested that they encyst in a second intermediate host. Accordingly, various invertebrates were exposed to active cercariæ to find out whether penetration and encystment would occur.

TABLE I

Measurements of Cercariæum lintoni. All measurements are given in millimeters, and each is the average of several measurements. The figures of Linton and Shaw are for living specimens, while those of Miller and Northup are for fixed specimens.

	Linton	Miller and Northup	Shaw
Length	.327	.23	.355
Width, anterior	.06		
" middle	.123	.084	.118
" posterior	.06		
Oral sucker (diameter)	.043		.05
Ventral sucker (diameter)	.0623		.07
Excretory vesicle, length			.07
" " width			.045
Pharynx, length			.025
" width			.022
Stylet, length	.02		.0186

MORPHOLOGY OF THE LARVÆ

Sporocyst Stage

The appearance, form, and size of the sporocysts were reported by Linton. He found them to average 0.645 mm. in length, 0.272 mm. in width, and to contain 2 to 40 cercariæ per sporocyst. My observations are in substantial agreement. There are about 18 to 20 cercariæ in a medium-sized sporocyst and their arrangement is shown in Fig. 3. Encystment of the cercariæ within the sporocyst was never observed.

Cercariæum

As noted by Linton, the cercariæ manifest much difference of form in extension and contraction. They are elongate forms, usually ovate, with the greatest width at the region of the ventral sucker or just posterior to it. Usually the anterior end narrows gradually and the posterior tip is slightly angular. A specimen full extended measured 0.83 mm. in length and 0.055 mm. in greatest width, while in a contracted condition it was only 0.25 mm. long. Table I gives measurements of the larva and certain of its structures as recorded by different authors.

The entire surface of the body is covered with minute spines; those of the anterior region are somewhat larger than those farther posteriad. They are set in rows, approximately 250 in number, arranged in alternate fashion as indicated in Fig. 9. Linton reported that the anterior spine (stylet) is not easily seen in mounted specimens, while Miller and

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PLATE I

Explanation of Abbreviations in Figures

c—cercariæum
cp—cirrus pouch
dg—ducts of cephalic glands
e—esophagus
ec—encysted cercariæum
ed—ejaculatory duct
es—excretory sphincter
et—lateral excretory tubule
ev—excretory vesicle
g—cephalic gland
gl—gland posterior to ventral sucker
gp—genital pore
ic—immature cercariæum
icw—inner cystic wall
inc—intestinal cecum
m—metraterm

o—ovary
ocw—outer cystic wall
og—opening of cephalic gland duct
ogl—opening of duct of gland posterior to ventral sucker
os—oral sucker
ov—oviduct
p—pharynx
pp—prepharynx
s—stylet
sr—seminal receptacle
sv—seminal vesicle
t—testis
u—uterus
vd—vas deferens
vs—ventral sucker

Explanation of Figures

Fig. 1. *Cercariæum lintoni* Miller and Northup, ventral view.

Fig. 2. Anterior portion of *C. lintoni* showing the relationship of the stylet and the ducts of the cephalic glands when the stylet is drawn within the oral sucker.

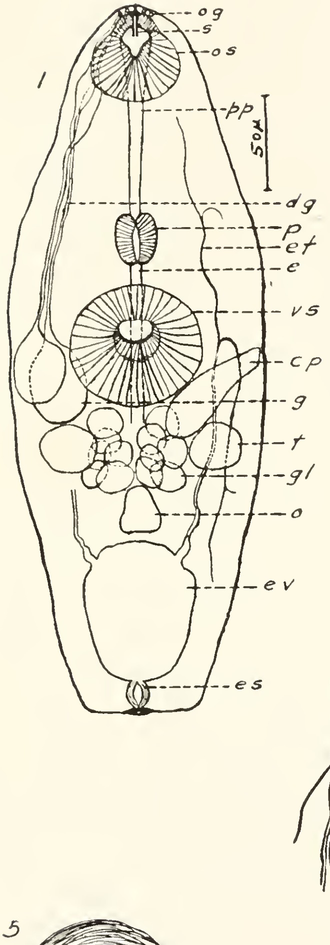
Fig. 3. Diagram of a sporocyst of *C. lintoni*. Note the relative positions of mature and immature cercariæ.

Fig. 4. Diagrammatic drawing of a parapodium of *Nereis virens* Sars., showing the characteristic location of cysts.

Fig. 5. Camera lucida drawing of a ten-day-old cyst of *C. lintoni*. Note the two layers of the cyst wall.

Fig. 6. Camera lucida drawing of the same ten-day-old (Fig. 5) cyst with the cercariæum in a different position.

Plate I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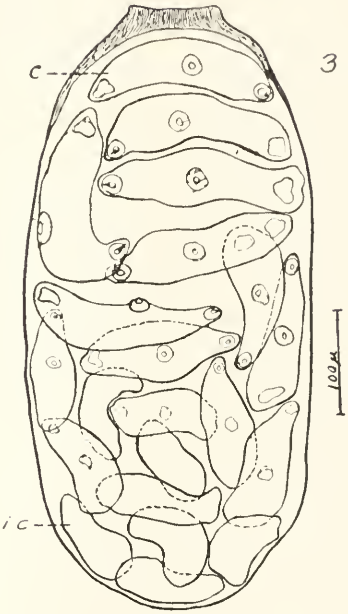
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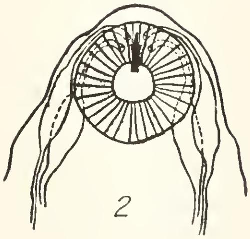
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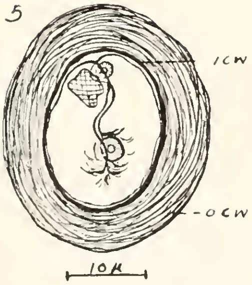
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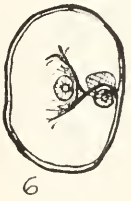
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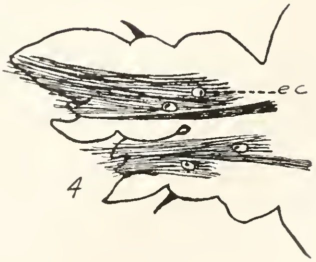
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Northup merely sketched it in their figure without mentioning it in their description. It is prominent in all active, living specimens and is shown, much as Africa (1930) figured it, in Figs. 1 and 2.

All accounts of species agree as to the nature and location of the prepharynx and pharynx, but there is considerable disagreement as to the intestine. Linton stated that, " intestinal rami were not distinctly shown " while Miller and Northup reported " very narrow intestinal ceca, traceable only in serial sections, reaching almost to the excretory vesicle." Miller and Northup, and Africa, figure a bifurcation of the short esophagus at the anterior margin of the ventral sucker and slender intestinal ceca which extend caudally. The writer was unable to distinguish such intestinal ceca, and study of later stages indicates that they probably do not exist.

The description of the excretory system, begun by Linton and emended by Miller and Northup, was completed by Africa. He gave the excretory formula as $2[(2+2)+(2+2)]=16$ flame cells, and figured the flame cells with connecting tubules. My observations are in full accord and verify his conception of certain connecting tubules of which he was not positive.

Rudiments of the reproductive organs were observed by Linton and also by Miller and Northup. Linton's observation concerning the testes is confirmed but the structures he identified as the rudimentary ovary and the " beginning of the uterus " have been shown in the present study to be the beginnings of the cirrus sac and the ovary respectively. He suggested that certain granular masses might be the " beginnings of diffuse vitellaria," but this point is still uncertain.

The two pairs of cephalic glands shown in Fig. 1 have been described by previous workers. But in addition, by intra-vitam staining with neutral red, I have been able to recognize six pairs of glands in the region just posterior to the ventral sucker.

Experimental Studies

Environmental Effects

The effects of various dilutions of sea water on the activity and longevity of these cercariæ were reported (Stunkard and Shaw, 1931). Specimens placed in 50 per cent and 25 per cent sea water remained alive as long or even a few hours longer than those in undiluted sea water. Room temperature was used in all of these experiments. The cercariæ lived, under these conditions, for 36 to 70 hours.

The cercariæ were much more resistant than those of other species, such as *Cercaria quissetensis* Miller and Northup (from the same host), on exposure to low temperatures. A dish containing hundreds of recently emerged specimens was placed at a temperature of 4° to 6° C. The early reaction was an increase in activity, but an hour later they were less active and there was a gradual decrease of activity during the remainder of the lifetime. By the eighth day a few had died, by the tenth day the majority were dead, and only a few showed slight activity on the twelfth day. The same experiment with *Cercaria quissetensis* showed that species to be much less resistant; each cercaria had ceased all activity by the end of the fourth day.

Infection Experiments

Since the cercariæ are tailless, they are unable to swim and an intermediate host must be present in the immediate vicinity of the snail from which they emerge. Miller and Northup described their activity as, "inch-worm locomotion . . . by successive attachment of posterior end of body, modified by invagination into an adhesive disc, then extension of body and attachment by ventral sucker." Very rarely one was found that had encysted on the side of the dish, but usually they showed gradual decrease in activity unto the point of death.

Nereis virens Sars. was successfully infected in the first attempt and gave positive results in all later experiments. The worm was placed in a bowl of sea water containing several hundred recently emerged cercariæ. After an exposure of from two to five hours it was removed to a specimen bowl, which contained well washed sand one-half inch deep. The bowl was then covered with a piece of scrim cloth held in place by a rubber band and placed under a slowly running stream of sea water. In this manner the infected worm could be kept for several weeks. It was interesting to note that cysts were never found in any part of the host other than the parapodia. Their typical location in the parapodium is indicated in Fig. 4; no doubt the rich blood supply in this region affords especially favorable conditions for development of the metacercariæ. At frequent intervals, parapodia were clipped off and the cysts dissected out and studied. The metacercariæ were removed by slightly crushing, or dissecting the cysts, and studied by the same methods used for the cercariæ.

Various invertebrates, including other annelids (*Hydroides* sp., *Lumbrinereis hebes, Scoloplos robustus,* and *Arabella opalina*) [1] and the flatworm, *Bdelloura candida,* were used in similar studies. These species were selected since they may occur in the same habitat as *Nassa obsoleta* and conceivably might serve as hosts of the parasite. In each instance the cercariæ crawled over the surface of the exposed animal and a few penetrated the body wall and encysted. Such cercariæ, when removed from their cysts, did not manifest the activity and developmental changes that characterize metacercariæ dissected from cysts in *Nereis virens*. In no other host did development occur; on the contrary the larvæ died and the cysts became brown and opaque, according to numerous examinations.

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[1] The author is endebted to Dr. L. P. Sayles, Department of Biology, College of City of New York, for identification of the last three forms.

To observe the method of penetration, parapodia were cut from noninfected *Nereis* and placed in Syracuse watch glasses with many active cercariæ. In a few moments the cercariæ were crawling over the parapodia. An hour later several were in very much contracted form and seemed to be inactive but attached to the parapodia. A short time later some of these worms, with anterior end against the parapodium, showed actual "pecking" and boring movements. At the end of two hours some had the anterior end well buried in the parapodium with the caudal end showing great muscular activity. At this time one was observed to be buried in the tissue with only the extreme posterior tip free. One hour later, examination with the binocular showed some within the parapodia and with walls around them. Since the parapodia began to show signs of disintegration at this point the study was not carried farther. It seems quite evident that the cercariæ actually bore their way into the fleshy parapodia by means of the stylet, and presumably the secretion of certain glands, notably the cephalic glands, aids in the process.

Nereis virens has proved to be an extraordinarily convenient host for such a study. As stated above, the experimental method of infestation was very simple since all that was necessary was to isolate a worm in a dish with hundreds of active cercariæ for two to five hours. Each time such an exposure was made the majority of parapodia, examined later, contained from one to six cysts—the results of the penetration and encystment of as many cercariæ. The infected *Nereis* could be kept alive for weeks and did not seem to suffer from the periodic clipping of its parapodia.

Development of the Metacercaria in Nereis

The development of the metacercaria within its cysts is typical, since there is a gradual reduction of larval structures with concomitant growth and differentiation of the organs of the adult stage, which is yet unknown. The transformation of the larva from cercaria to metacercaria is shown in Figs. 6, 7, 8, 10, and 11.

The Cyst

The cysts may be readily observed in the parapodia with a binocular microscope. The wall of the cyst is quite transparent in early stages and never becomes truly opaque, even in later stages, as long as the metacercaria is alive. In older stages the cyst wall consists of two coats, as shown in Fig. 5. The inner coat, which is formed from glandular material of the larva, is thin, clear, and easily ruptured. Around it there develops a thick coat of connective tissue, produced as a reaction of the surrounding tissues of the parapodium, which is tough and difficult to break. Pressure on the cyst causes the contained worm to squirm and it is possible to observe certain features very clearly. The excretory vesicle is especially prominent, as it is filled with concretions that make it appear dark. The cysts increase in size as the metacercariæ develop; Table II summarizes measurements made of cysts of various ages.

Loss of Larval Structures

The large cephalic, or penetration glands are resorbed and have disappeared entirely by the sixth day. The stylet is clearly visible and apparently unchanged during the first ten days of the encysted stage. By the thirteenth day it is reduced in size and the form is somewhat modified as the tip is very blunt. In the cercarial stage the stylet measures 0.0186 mm. in length and 0.033 mm. in width. In the thirteen-day-old metacercaria it measures 0.0156 by 0.0022 mm.; in the twenty-two-day-old 0.0066 by 0.002 mm.; and in the twenty-six-day-old the stylet is lacking completely.

TABLE II

Measurements of cysts of Cercariæum lintoni M. and N. which had been dissected out of Nereis. Each figure is the average of eight or more measurements made of forms in an uncovered drop of water.

Age	Length	Width
days	*mm.*	*mm.*
4	.15	.13
13	.21	.155
26	.246	.202
36	.258	.191
45	.26	.225

Development of Adult Characters

Certain structures, e.g. the details of the excretory system, are established in the cercarial stage and seem to remain constant throughout development. But the digestive system and the reproductive organs become more developed, or at least more evident, as the metacercariæ increase in age and size.

As discussed previously, the exact nature of the digestive system in the cercarial stage is doubtful. The prepharynx is long with the pharynx just anterior to the ventral sucker. The first positive observations of the post-pharyngeal region of this system obtained by the writer were in the study of a living ten-day-old metacercaria, in which it was possible to see a single tube extending posteriorly. Liquid, with suspended granules, was being forced back and forth in this tube. In the study of metacercariæ 30 days old and older, it was easy to see the exact nature of the entire system (see Fig. 11) in both living and mounted specimens. The esophagus extends posteriorly, dorsal to the gonads, and in the region posterior to the cirrus pouch it gives rise to two large bulb-like intestinal ceca.

The reproductive organs, as they appear in metacercariæ 35 to 49 days old, are shown in Fig. 11. The testes are located exactly as Linton described them in the cercariæum. The vasa deferentia are short, slightly coiled tubules leading to the cirrus pouch, in which they unite to form a seminal vesicle. From the vesicle an ejaculatory duct traverses the cirrus sac and the genital pore is situated on the left margin of the body at the level of the aperture of the acetabulum. The ovary is situated just anterior to the excretory vesicle, as reported by Miller and Northup. The oviduct is short and communicates with a seminal receptacle on the right side of the body, while the uterus extends as a long tortuous canal to the metraterm which is parallel to the cirrus sac and opens at the genital pore. The vitellaria were not located with certainty.

Measurements of metacercariæ of various ages (dissected from their cysts) are given in Table III. These measurements were used in part in constructing Figs. 10 and 11. Comparison with Table I shows the extent of development.

Adult Host

Only one attempt was made to determine the host of the adult trematode and this experiment was unsuccessful. The host of the

---

EXPLANATION OF PLATE II

FIG. 7. A diagrammatic sketch of a ten-day-old metacercaria emerging from its cyst which has been crushed by slight pressure.

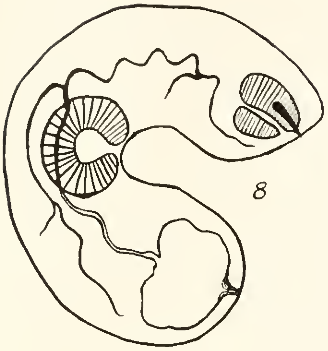
FIG. 8. Lateral view of a two-day-old metacercaria of *Cercariæum lintoni*, showing the relationship of the most prominent structures of that stage.

FIG. 9. Diagram to show the plan of arrangement of the spines which cover the surface of the body of *C. lintoni* and its metacercaria.

FIG. 10. Diagrammatic drawing of 18-day-old metacercaria of *C. lintoni*. Ventral view.

FIG. 11. Diagrammatic drawing of 45-day-old metacercaria of *C. lintoni*. Ventral view.

PLATE II



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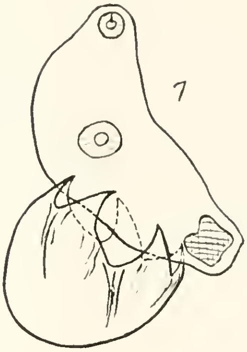

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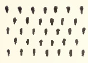

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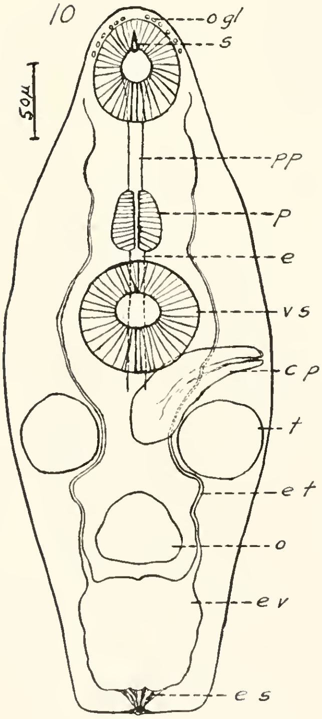

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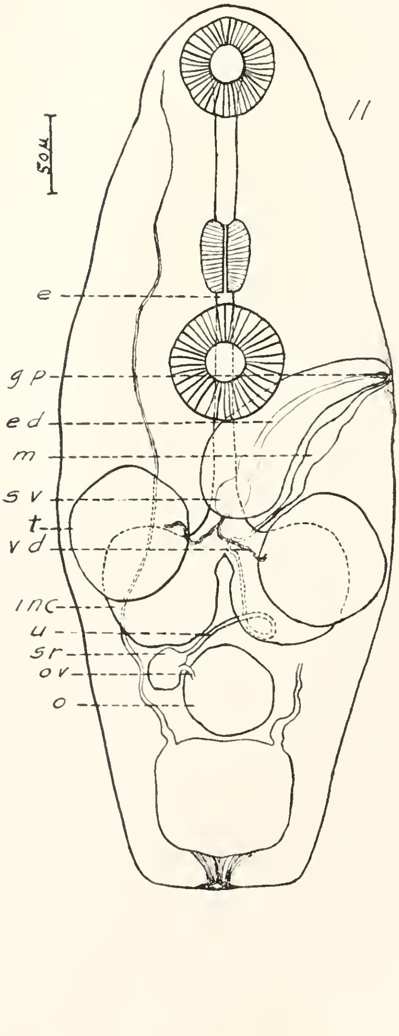

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adult is, presumably, a fish which eats *Nereis*. Since the eel and *Nereis* have been observed in the same habitat in the Woods Hole region, since eels are believed to eat clam worms, and since the eel is a hardy animal which will withstand laboratory conditions, this species was selected for the experiment. On August 7, 1931 two infested *Nereis virens* (one containing 45- and the other 38-day-old metacercariæ) were fed to an eel, *Anguilla chrisypa* Raf. The eel died three weeks later but no trematodes were found.

DISCUSSION

When it was discovered that *Cercariæum lintoni* would develop in *Nereis virens,* it appeared probable that advanced metacercarial stages would be sufficiently mature so that comparison of them with descriptions of marine trematodes might suggest the adult form. It was thought that at least the family to which they belong could be determined. This hope has not, however, been realized. Examination of the papers by Looss (1894), Lühe (1909), Odhner (1911), Linton (1898, 1899, 1904, 1907, 1910), Manter (1926, 1931), and Fuhrmann (1928) has failed to disclose a trematode that could be considered as the adult form of *C. lintoni.*

TABLE III

Measurements of metacercariæ of Cercariæum lintoni *M. and N.* All measurements are in millimeters; all measurements made from living specimens.

	13 days old	18 days old	27 days old	45 days old
Length	.42	.46	.47	.575
Width	.15	.1725	.24	.215
Oral sucker, length	.075	.07	.07	.065
" " width	.06	.06	.0625	.06
Ventral sucker, length	.07	.075	.075	.075
" " width	.085	.08	.075	.075
Pharynx, length	.035	.04	.042	.0484
" width	.032	.035	.038	.0374
Testis, length	.065	.06	.065	.08
" width	.06	.06	.07	.085
Ovary, length	.05	.05	.055	.06
" width	.06	.06	.06	.06
Excretory vesicle, length	.075	.075	.07	.075
" " width	.07	.08	.09	.09
Stylet, length	.0156	.0143	.0066	—
" width	.0022	.0022	.002	—

Palombi (1930) worked out the life history of a tailless cercaria at Naples and found that it developed into *Diphterostomum brusinæ* (Stossich), a member of the family Zoogonidæ. He compared the cercaria of

D. brusinæ with *Cercariæum lintoni* and concluded that they were closely related species. He stated: "*Cercariæum lintoni* Miller e Northup parassita die *Nassa obsoleta* (Say), presenta sensibili affinitá con la cercariæ di *Diphterostomum brusinæ* Stoss. parassita di *Nassa mutabilis* L., *N. reticulata* L. e *Natica Poliana* Delle Chiaie. Non e improbabile che essa rappresenti la forma larvale die una specie della famiglia *Zoogonidæ* Odhner." Table IV gives a detailed comparison of the two species and indicates that they are not as closely related as Palombi believed.

TABLE IV

Comparison of the cercariæum of Diphterostomum brusinæ Stossich and Cercariæum lintoni Miller and Northup

	Cercariæum of *D. brusinae* Stoss.	*Cercariæum lintoni* M. and N.
Length	.23 mm.	.32 mm.
Width	.10 mm.	.102 mm.
Size of ventral sucker	Considerably larger than oral sucker.	Slightly larger than oral sucker.
Position of ventral sucker	Just posterior to middle region.	In middle region.
Lateral view of v. sucker	Shows two peculiar "prominences."	Without peculiar prominences (Fig. 8).
Stylet	Present.	Present.
Pre-pharynx	None.	Long.
Pharynx	Just posterior to oral sucker.	Just anterior to ventral sucker.
Esophagus	Long.	Rather long.
Intestinal ceca	Slender and short.	Short, bulb-like.
Testes	Just posterior to ventral sucker.	Just posterior to ventral sucker.
Genital pore	Median, slightly anterior.	Lateral, left, just posterior to ventral sucker.
Sporocyst	In *Nassa mutabilis*.	In *Nassa obsoleta*.
Free living stage	None.	Emerges from snail; active for hours.
Encystment	In snail host; in or out of sporocyst.	In parapodia of *Nereis virens*.
Mode of entrance into next host after snail	Eaten by fish.	Penetrate parapodia of *Nereis virens*.

Palombi suggested that *Cercariæum lintoni* may be the cercarial form of a species of *Deretrema* Linton, Family *Zoogonidæ*. The morphological characters of the metacercaria show, however, that the species does not belong to the genus *Deretrema*. In the arrangement of reproductive organs it agrees with members of the family *Zoogonidæ*, but the digestive system is very different. The digestive system of the metacercaria resembles that of *Saccocoelium* Looss (Family *Haploporidæ*), but the reproductive organs do not correspond to those of the *Haploporidæ*. It is, accordingly, impossible to assign *C. lintoni* to any family as they are at present characterized, and determination of the systematic position of the species must await further knowledge.

SUMMARY

The description of *Cercariæum lintoni* Miller and Northup as given by earlier workers is supplemented and emended in the light of knowledge obtained by comparing the cercariæum with its metacercarial stage, developed in experimental animals. The true nature of the digestive and reproductive systems is made clear.

Cercariæum lintoni penetrated the parapodia of *Nereis virens*, encysted, and developed into the metacercarial stage. Although the cercaria would penetrate and encyst in other invertebrates, the clam worm was the only form in which development was secured. *Nereis virens* appears to be a natural host. The metacercaria develops adult features as the larval characters disappear.

It is difficult to determine the relationship of this species to other trematode groups; the digestive system is similar to that of the species of the family *Haploporidæ*, but the arrangement of the reproductive organs is very different and resembles that of the *Zoogonidæ*. Palombi's suggestions concerning the relations of the species are discussed.

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HEMOLYTIC ACTION OF SILVER OCCURRING AS AN IMPURITY IN CHEMICALLY PURE SODIUM CHLORIDE[1]

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The hemolysis of fish erythrocytes when placed in isotonic solutions made from certain brands of sodium chloride C.P. has been reported by Williams and Jacobs (1931). The experimental results presented by these investigators suggest an impurity in the salt as the causative agent. Since sodium chloride is the main constituent of most isotonic mediums used in biological experiments it seemed of importance to determine the exact nature of this toxic principle. Data will be presented in this paper to show that the hemolysis of fish erythrocytes by isotonic solutions made from certain brands of so-called chemically pure sodium chloride is due to the presence of small amounts of silver occurring as an impurity in the salt.

PROCEDURE

Fish were bled directly from the ventral aorta into a beaker. The blood was defibrinated and the fibrin removed by filtering through dry cheesecloth that had been washed in distilled water. The blood was then centrifuged for five minutes at a rate just sufficient to cause separation of the cells and serum and the cell volume roughly adjusted when necessary to the normal value of 25 per cent so as to insure uniformity in the cell content of blood obtained from different fish. This procedure was found to be necessary as the rate of hemolysis was affected by the amount of red cells used. One drop of the remixed blood was added from a bulb pipette, constructed so as to deliver uniform drops (1 cc. = 28 drops), to 25 ml. of the isotonic (0.25 M) sodium chloride solutions contained in test tubes (200 × 30 mm.). The progress of hemolysis with time was followed by visual inspection.

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[1] Presented before the American Society of Biological Chemists at Philadelphia, April 28–30, 1932.

[2] With the coöperation of Harold Blumberg, Department of Biochemistry, Johns Hopkins School of Hygiene and Public Health.

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RESULTS

The blood cells of different species of fish show a wide variation in their resistance to hemolysis when placed in isotonic solutions of various brands of sodium chloride. In Table I are assembled representative times for complete hemolysis of the blood cells of a number of species of marine fish when placed in isotonic solutions made from four different brands of sodium chloride. The blood cells of both the mackerel and the cunner are rapidly hemolyzed. Since both of these species were easily obtainable and afforded a rapid means of testing for the toxic principle they were used throughout in the work that follows. (Table VI excepted.)

TABLE I

Hemolysis time for the erythrocytes of various species in isotonic sodium chloride

Species	Brand 1	Brand 2	Brand 3	Brand 6
Cunner (*Tautogolabrus adspersus*)	No hemolysis	5 min.	6 min.	7 min.
Tinker Mackerel (*Scomber scombrus*)	No hemolysis	5.5 min.	7.5 min.	27 min.
Bull's Eye Mackerel (*Scomber colias*)	No hemolysis	3 min.	4 min.	5 min.
Butterfish (*Poronotus triacanthus*)	No hemolysis	4 min.	5 min.	10 min.
Flounder (*Platessa dentatus*)	No hemolysis	10 min.	10 min.	15 min.
Scup (*Stenotomus chrysops*)	No hemolysis	18 min.	32 min.	
Tautog (*Tautoga onitis*)	No hemolysis	22 min.	34 min.	
Common Sea Robin (*Prionotus carolinus*)	No hemolysis	25 min.	25 min.	30 min.
Rudder Fish (*Palinurichthys perciformis*)	No hemolysis	25 min.	25 min.	20 min.
Dogfish (*Mustelus canis*)	No hemolysis	75 min.	60 min.	

Of the four brands shown in Table I only No. 1 was non-toxic. This is a Kahlbaum's salt, which agrees with the findings of Williams and Jacobs. This salt is the only one which is not mined. It is prepared from sodium carbonate and hydrochloric acid in porcelain vats. This fact would immediately lead one to suspect the occurrence of an impurity in the other salts. However, when seeking the cause for toxic actions of single salt solutions on biological material it is always necessary to keep in mind the action of so-called antagonistic ions. That the lack of antagonistic ions cannot be the explanation in this case is shown by the results presented in Table II.

An isotonic solution of sodium chloride which showed rapid hemolysis was progressively diluted with sea water that had also been made isotonic by dilution and which caused no hemolysis. Since sea water contains a suitable mixture of the ions deemed necessary to form a physiologically balanced solution, such admixture should result in the complete disappearance of the toxic action. However, as can be seen from the data presented, the toxic effect persisted, though diminished, even when sea water formed as much as 80 per cent of the mixture. Moreover, almost identical results were obtained when the dilution was made with a non-toxic sodium chloride solution. Thus the presence of calcium, magnesium, and potassium had no effect on the toxic action. Therefore, it seemed reasonable to conclude that the toxic action was caused by an impurity.

Table II

Effect of dilution of toxic salt solution with isotonic sea water.
0 = no hemolysis; ++++ = complete hemolysis.

Isotonic* sea water	0 ml.	5 ml.	10 ml.	15 ml.	20 ml.	25 ml.
NaCl No. 2	25 ml.	20 ml.	15 ml.	10 ml.	5 ml.	0 ml.
Hemolysis Time *min.*						
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	++	+	0	0	0	0
5	++++	+	0	0	0	0
6		++	+	0	0	0
7		++++	++	0	0	0
8			++	0	0	0
9			+++	+	0	0
10			++++	++	0	0
15				++++	0	0
100					++++	0

* Similar results obtained when a non-toxic salt solution substituted for isotonic sea water.

In order to determine whether this impurity present in sodium chloride was organic or inorganic, samples of salts that showed hemolytic effects were fused in a platinum crucible and held in the liquid state for at least fifteen minutes. Such treatment should destroy, at least partially, any organic matter present. Hence if the impurity is organic in nature, isotonic solutions made from the fused salt should cause little or no hemolysis whereas the toxic action should remain undiminished if the impurity is inorganic.

As shown in Table III, fusing caused no diminution in the toxic action of a salt; in fact, an increase in toxicity seemed to have occurred. Hence the impurity was classed as an inorganic substance. The only reasonable explanation for the increased toxicity after fusion seemed to be the formation of oxides with an attendant increase in alkalinity of the resulting solution. Rough tests with indicators showed that such was the case, suggesting that the toxic action might vary with pH.

TABLE III

Hemolytic action of various salts before and after fusion.

S.G. = swirl gone—disappearance of characteristic swirl shown by cells on shaking.

Salt*	1	1 Fused	2	2 Fused	3	3 Fused	4	5	6
Hemolysis Time *min.*									
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	S.G.	++	0	++	0	0	0
4	0	0	++	++++	S.G.	++++	0	0	0
5	0	0	++++		S.G.		0	0	S.G.
6	0	0			+		0	0	+
7	0	0			++		0	0	+++
8	0	0			+++		0	0	++++
9	0	0			++++		0	0	
10	0	0					0	0	
300	0	0					0	0	

* Each number represents a different brand of sodium chloride, except No. 5 which was prepared from recrystallized sodium carbonate and hydrochloric acid.

In the hope that some light might be thrown on the nature of the inorganic impurity present in the sodium chloride, a study of the effect of pH on the rate of hemolysis was made. The results of one such experiment are shown in Table IV. The pH was controlled by adding 2 ml. of buffer to 23 ml. of isotonic salt solution, a procedure which altered the isotonicity but little. The difference in pH produced by dilution of the buffer was not detectable colorimetrically. As the pH increases, the rate of hemolysis also increases. Below pH 5.0 the hemolytic action is very slow and if present is masked by a change produced on the cells by the acid solutions. This effect of pH sug-

Table IV

Effect of pH on Hemolysis Time.

pH	3.0		4.0		5.0		6.0		7.0		8.0		9.0		10.0	
Salt*	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Hemolysis Time *min.*																
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0	++
3	0	0	0	0	0	0	0	0	0	+	0	+++	0	++++	0	++++
4	0	0	0	0	0	0	0	0	0	+++	0	++++	0		0	
5	0	0	0	0	0	0	0	+	0	++++	0		0		0	
10	0	0	0	0	0	+	0	++++	0		0		0		0	
15	0	0	0	0	0	++++	0		0		0		0		0	
30	†	†	†	†	0		0		0		0		0		0	

* All toxic salts showed this pH effect.

† Cells appear to have undergone a change, though not hemolysis.

gested as a working hypothesis, since the impurity was inorganic, that the causative agent was a cation combining with the protein of the cell wall only on the alkaline side of its isoelectric point. That this viewpoint of the mechanism may be entirely erroneous is fully recognized, but as a working hypothesis in the identification of the impurity it proved useful.

It now seemed reasonable to suppose that since all the brands of sodium chloride that contained this impurity came from a natural source, the unpurified or table salt would contain larger quantities of the toxic inorganic substance. Isotonic solutions made from several samples of table salt were found to be turbid because of the ingredients added to prevent caking. On filtering these solutions, it was found that they caused no hemolysis of fish erythrocytes. A possible explanation for this seemed to be that the toxic principle had been adsorbed and removed by the fine particles that had been filtered off. Though this proved not to be the case, as will be shown later, it suggested attempting to remove the impurity from the toxic solutions by adsorption. Animal charcoal seemed to be the best adsorbent to use, since if the inorganic impurity was adsorbed it could be concentrated by subsequent ashing of the charcoal.

Table V

Removal of Impurity by Adsorption on Activated Charcoal and Recovery on Ashing

Salt	1	1N*	2	2N	3	3N	6	6N	1A†	1B‡
Hemolysis Time *min.*										
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	+	0	0	0
4	0	0	++	0	+	0	++	0	0	0
5	0	0	++++	0	++	0	+++	0	S.G.	0
6	0	0		0	+++	0	++++	0	S.G.	0
7	0	0		0	++++	0		0	+	0
10	0	0		0		0		0	++++	0
200	0	0		0		0		0		++++

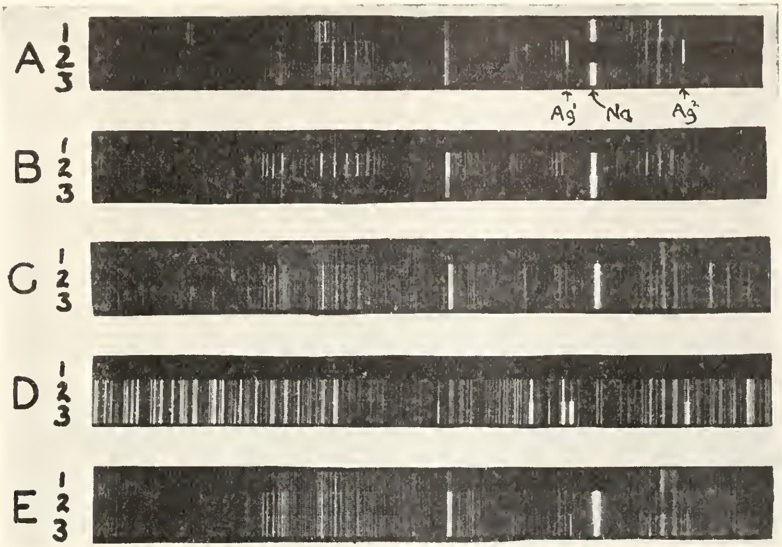
* N designates salt solution shaken with activated charcoal (Norite).

† NaCl No. 1 plus the ash of charcoal that had been shaken with a toxic salt solution.

‡ NaCl No. 1 plus the ash of untreated charcoal.

The results given in Table V show that when a toxic solution of sodium chloride is shaken with charcoal and the charcoal removed, the resulting solution has lost its toxic action. If this charcoal is now ashed, the ash treated with HCl, neutralized, and a small portion added to a non-toxic solution of NaCl, hemolysis occurs. The ash of untreated charcoal also shows a faint hemolytic action though very unlike the pronounced action of the treated charcoal.

Since the adsorption of the impurity on charcoal furnishes a simple means of purifying sodium chloride solutions for future use, it may be worthwhile to give in detail the procedure followed. To each liter of isotonic salt solution, two grams of animal charcoal (Norite) were added and shaken intermittently throughout the day. The solution



EXPLANATION OF PLATE I

Ag¹—Silver line λ3280.67 A.U.
Ag²—Silver line λ3382.89 A.U.
Na—Sodium line

A
1. Woods Hole Sea Salt (non-toxic)
2. $AgNO_3$
3. NaCl Brand No. 3 (toxic)

B
1. Graphite Electrodes
2. NaCl Brand No. 4 (non-toxic)
3. NaCl Brand No. 2 (toxic)

C
1. Graphite Electrodes
2. Table Salt No. 1 (non-toxic)
3. Table Salt No. 2 (non-toxic)

D
1. Graphite Electrodes
2. Ash of untreated charcoal
3. Ash of charcoal shaken with NaCl No. 2

E
1. Graphite
2. NaCl No. 3 made non-toxic by shaking with charcoal
3. NaCl No. 3 (toxic)

Obtained with Hilger E1 Quartz Spectrograph, using graphite arc and with technique similar to that described by Shipley, Scott, and Blumberg (1932).

was then allowed to stand overnight and the charcoal filtered off in the morning, the filtrate then being ready for use. The removal of the impurity seems to be a slow process as the addition of more charcoal with more frequent shaking does not enable one to obtain a non-toxic solution in shorter time. It is possible that a small amount of sodium chloride is also removed by the charcoal, which would change slightly the concentration of the solution. This change would be significant only where extremely accurate work is contemplated. Purification of the salt by recrystallization is ineffective.

Since a concentration of the toxic material was now possible by adsorption on charcoal and subsequent ashing, a spectrographic identification of the inorganic impurity seemed possible. Attempts to duplicate the hemolytic action by addition of various inorganic salts to non-toxic solutions had failed.

The results obtained with the spectrograph are shown in Plate I[3] and indicate that silver is the impurity present in the sodium chloride that causes hemolysis of erythrocytes. Those brands of salt that showed hemolysis, Nos. 2 and 3, give the *raies ultimes* of silver. Woods Hole sea water and Brand No. 4, which do not cause hemolysis, contain no detectable silver. It was also impossible to detect silver in two brands of table salt that were non-hemolytic. It had previously been thought that isotonic solutions made from these salts were non-hemolytic due to the removal of the toxic principle by adsorption on insoluble matter contained in them which was filtered off before use.

A comparison of the spectrographs of the ash of charcoal that had been shaken with a toxic salt solution and of the ash of untreated charcoal show the silver lines to be much brighter in the former than in the latter. The presence of silver in the untreated ash explains the delayed hemolysis that resulted on the addition of this ash to a non-toxic salt solution (see Table V). Finally, a comparison of the spectrographs of a toxic sodium chloride before and after shaking with charcoal shows that the silver lines have completely disappeared due to the charcoal treatment. These results would seem to indicate that in whatever state silver exists in the sodium chloride solutions, it is readily adsorbed by activated charcoal, and that the presence of silver is always attended by hemolytic action.

It remained to be shown that hemolysis could be produced by the addition of small amounts of silver to a non-toxic salt solution. Since the marine fish that had been used in the first part of this investigation were not obtainable in Baltimore, it was necessary to use freshwater fish. Several specimens of trout and perch were obtained from the

[3] Run by Mr. Blumberg.

State Hatcheries.[4] The erythrocytes of both these species were hemolyzed rapidly both by a toxic salt solution and by a non-toxic salt solution plus a small amount of silver nitrate. A typical experiment is shown in Table VI.

It can be seen that when silver nitrate is added to a non-toxic salt solution to give a final concentration of added silver equal to 10^{-5} molar, hemolysis occurs in about the same time as in a toxic salt solution. This added silver can then be removed by shaking with charcoal and a non-hemolytic solution obtained. Solutions stronger in silver than 10^{-5} molar are not obtainable, as a precipitate of silver chloride results. Solutions made hemolytic by the addition of silver show the same reaction to pH as a toxic salt solution (see Table IV).

TABLE VI

Hemolysis of Trout Erythrocytes by Silver

Solution number	1	2	3	4	5
	NaCl No. 2 0.15 M	Solution 1 shaken with charcoal and filtered	Solution 2 plus $AgNO_3$ to give $[Ag] = 10^{-5}$ M	Solution 2 plus $AgNO_3$ to give $[Ag] = 10^{-6}$ M	Solution 3 shaken with charcoal and filtered
Hemolysis Time *min.*					
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	S.G.	0	0
4	S.G.	0	+	0	0
5	++	0	++	0	0
6	+++	0	+++	0	0
7	++++	0	++++	0	0
8		0		0	0
9		0		0	0
10		0		S.G.	0
15		0		+	0
20		0		+++	0
30		0		++++	0

This leaves little doubt that silver is the toxic agent in sodium chloride that causes hemolysis of fish erythrocytes. From a comparison of the rates of hemolysis in a toxic salt solution and a non-toxic salt solution plus known amounts of silver, the concentration of silver in various brands of sodium chloride is found to be of the order of magnitude of 10^{-3} to 10^{-4} per cent by weight.

Other inorganic salts were tested for their hemolytic action. The following salts were added to a non-toxic salt solution to produce concentrations ranging from 10^{-3} to 10^{-6} molar: $AlCl_3$, $AuCl_3$, $BaCl_2$,

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[4] Through the courtesy of Mr. Swepson Earle, Commissioner.

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$BeCl_2$, $CaCl_2$, $CdCl_2$, $CeCl_4$, $CoCl_3$, $CrCl_3$, $CsCl$, $CuCl_2$, $FeCl_3$, $HgCl_2$, KCl, $LaCl_3$, $LiCl$, $MgCl_2$, $MnCl_2$, NH_4Cl, $NiCl_2$, $PbCl_2$, $PdCl_2$, H_2PtCl_6, $RbCl$, $RhCl_3$, $RuCl_3$, $SbCl_3$, $SnCl_2$, $SrCl_2$, $ThCl_4$, $TiCl_3$, $TlCl$, $UO_2(NO_3)_2$, VCl_2, $ZnCl_2$. Only mercury and palladium showed marked hemolytic action. Hemolysis by the mercuric salt was always accompanied by a turbidity of the solution. The palladium salt produced a rapid hemolysis giving a clear solution at dilutions similar to that for silver. That palladium is not the impurity present in sodium chloride can be easily shown by the addition of sodium iodide. Concentrations of palladium (10^{-5} to 10^{-6} molar) that cause hemolysis at rates similar to that of toxic salt solution show a pronounced brown coloration on the addition of NaI due to the formation of insoluble palladium iodide. The addition of iodides to a toxic salt solution causes no coloration but does delay hemolysis, probably because AgI is formed, which is more insoluble than AgCl.

The striking similarity in hemolytic action of silver and palladium is of interest for these two elements lie in entirely different groups. Since silver and mercury have been widely used as bactericidal agents ~~if necessary~~, the possibility suggests itself that palladium might also be useful in this connection, especially since its chloride is so much more soluble than that of silver.

Discussion

In searching for a toxic inorganic impurity in sodium chloride, it is doubtful if silver would be among the first elements to be considered. The low solubility of silver chloride is a well-known fact, and according to the solubility product one would expect its solubility to be still lower in the presence of excess chloride ions. However, there is evidence in the literature to show that silver chloride tends to form soluble complexes in sodium chloride solutions. Pinkus and Berkolaïko (1930) have presented data to show that the solubility of AgCl increases as the concentration of sodium chloride rises. This fact, coupled with the knowledge that quantities of minute importance to the analyst are of extreme physiological significance, tend to lessen the incredibility of the toxic action of silver occurring as an impurity in sodium chloride.

As stated early in this paper, it was found that all brands of salt that showed hemolytic properties were mined. However, crude table salt which is also mined contains no silver, so that it seemed unlikely that the silver occurred in the natural deposits. After much inquiry it was found that the final step in the purification of crude mined salt for the chemical trade was carried out in silver-lined vessels because less corrosion occurred in such vessels. This then would appear to be the source of the silver that occurs in some brands of sodium chloride.

The presence of silver in sodium chloride as observed here is not a chance occurrence. In a private communication, Dr. E. Wichers states that Mr. A. Isaacs found silver to be present when testing sodium chloride for suitability as a chemical reagent. He also points out that the specifications for sodium chloride published by the American Chemical Society (1928) indicate that silver may be found.

Since presenting this paper before the Society of Biological Chemists, there has come to my attention several instances in which the employment of an artificial biological medium composed of sodium chloride in whole or in part has proved unsuitable. If the brand of sodium chloride used was changed, usually to Kahlbaums, which has been found silver-free, the medium became satisfactory in all respects. That the toxic action of certain brands of sodium chloride is not limited to the hemolysis of fish erythrocytes has been shown by Williams and Jacobs (1931). These workers have found that salts which cause hemolysis usually also exhibit a more toxic action on various other marine organisms. It has also been observed in this laboratory that not only fish but also mammalian erythrocytes undergo hemolysis when placed in isotonic solutions made from sodium chloride containing silver. In the case of mammalian red cells the rate of hemolysis is in terms of hours instead of minutes, though the final effect is none the less definite. Controls showed no hemolysis after several days.

It would, therefore, seem highly appropriate to sound a note of caution in the indiscriminate use of sodium chloride in the preparation of physiological saline solutions, no matter what may be their intended use. Though the effect of the silver, occurring as an impurity, on other biological material may not be as obvious as in the case of fish erythrocytes, it may nevertheless be as serious. Unseen changes in the biological material may have occurred which will alter entirely the response to experimental procedure. The use of silver-free sodium chloride solutions in such work is therefore suggested. Such solutions can easily be prepared by the simple method of purification outlined above.

SUMMARY

1. The hemolysis of fish erythrocytes by isotonic solutions made from certain brands of sodium chloride has been shown with the aid of the spectrograph to be due to silver occurring as an impurity in those salts.

2. The amount of silver present in such salts is of the order of magnitude of 10^{-3} to 10^{-4} per cent and originates from the use of silver-lined vessels in the purification process.

3. A simple procedure for the removal of silver from such salts by adsorption on charcoal has been described.

4. Palladium is the only other element that has been found to be as effective as silver in causing hemolysis.

5. Since the toxic action of silver is not limited to the hemolysis of fish erythrocytes, it is suggested that silver-free sodium chloride be used in the preparation of physiological saline solutions.

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THE RELATION OF THE THYROID AND THE HYPOPHYSIS TO THE MOLTING PROCESS IN THE LIZARD, HEMIDACTYLUS BROOKII

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The molting of the epidermis occurs with great regularity in many groups of lizards. In the Amphibia, where this periodicity is less regular, the thyroid has been shown to play an important rôle in the process (Adams, Richards, and Kuder, 1930). Complete cessation of molt has been reported in the common European lizard, *Lacerta*, following thyroidectomy (Drzewicki, 1928). Since the investigator experimented with only a small series of lizards and since we have observed that lizards in poor health are very irregular in their molting, it seemed highly desirable to repeat his work on a larger scale. We have selected for this purpose a lizard which molts at more frequent intervals than *Lacerta*. Environmental influences have been ruled out as far as possible by keeping both experimental and control series in a large incubator at a constant temperature and at a humidity which fluctuated only slightly. As already reported in a preliminary note (Noble and Bradley, 1931), we have obtained results very different from those Drzewicki reported. The present paper represents a detailed statement of our findings.

MATERIALS

The lizards we found most convenient to employ in this study were geckos of the species *Hemidactylus brookii*. Our entire series came from Samaná, Dominican Republic, where this species, imported from Africa, is now abundant. The lizards were maintained throughout our experiment in cages measuring 50 cm. long, 35 cm. deep, and 33 cm. wide, with glass sides, a removable iron bottom, and a screen top. A layer of sand, frequently changed, covered the floor of each cage, and the lizards were given ample hiding places among strips of cork bark introduced into the cages. From 15 to 20 lizards lived in each cage and since they rarely mutilated one another they appeared to have sufficient room. The lizards were fed with mealworms (*Tenebrio molitor*) and wax worms (*Galleria mellonella*) two, or rarely, three times a week. Fresh water was supplied daily. The series of cages were arranged in rows in a large, glass-sided incubator maintained at a constant temperature of 28° C. and average relative humidity of 53.7 per cent. A fan was provided to distribute the heated air evenly throughout the incubator.

The lizards were numbered and individual records made from day to day. Molting is more rapid in the Gekkonidae than in any other family of lizards. The head, body, and tail do not have independent molting cycles as in some iguanids. As the period of molt approaches the entire skin appears more opaque or gray. The superficial layers of epidermis are freed from the underlying layers over most of the lizard's body within a short time. Consequently, the lizard appears to be veiled over all its body with the white shed skin which always splits along certain lines of the body. The lizards rub off some of the skin but most of it they pull off with their jaws, usually swallowing it piece by piece. The lizards aid one another in this dispensing with the old skin. The habit is annoying to the observer because it may result in a complete removal of the skin within a few hours. Usually the molted skin is entirely shed within 24 hours after it first loosens from the body. The opaqueness of the skin to be shed is discernible one or two days before actual loosening occurs. A recently shed lizard has a bright integument very different from the opaque skin of the day preceding molt. Although in most cases some pieces of skin were found as evidence of a molt, in a few cases we had to rely on the change in skin opacity reported in our daily record as proof that a molt had occurred.

THE MOLTING CYCLE

From September 1930 to December 1931, we made molting records on 123 individuals of *Hemidactylus brookii*. We have 247 records of molt in this series of lizards which represents the control series of the several experiments to be detailed below. These 123 lizards molted on the average every 15.73 days. Our minimum period between molts was 11 days, our maximum apparently 23.2, these extremes being the normal rate for a very few healthy individuals, and almost all other records falling near the average. *H. brookii* is a tropical species and when maintained in good health at a constant temperature it shows a remarkable regularity in its molting periodicity.

EFFECT OF THYROIDECTOMY ON MOLTING

In January 1931, we selected a series of the lizards and removed their thyroids under the binocular microscope. It was found advisable to make an incision across the whole width of the throat immediately anterior to the clavicles. The muscles overlying the thyroid were either cut or spread apart. The several lobes of the thyroid were then gently freed of their attachment by means of a fine forceps and the blood vessels pinched off with the same instrument. In spite of the extent of the incision little bleeding occurred and this was never sufficient to cause death. After removing the thyroid, the muscles were drawn together with three or four separate stitches of fine silk thread, and the skin snapped together with skin clips. An antiseptic adhesive powder was shaken on the wound. By this time the lizards had usually recovered from the ether. Controls were opened in exactly the same way and the thyroid pulled away from the surrounding tissue sufficiently to cause some bleeding. Thyroidectomy apparently did not impair the health of the lizards. Immediately upon recovering from the effects of etherization they were as active as before, and they continued to behave as normal *Hemidactylus*. The death rate among these animals was no higher than that among normal ones.

Most of the lizards in the series were allowed to live until they died but many were killed after they had shed a sufficient number of times following the operation. The throats of the experimental lizards were sectioned serially and examined carefully for the presence of thyroid follicles. Some lizards died too soon to show any deviation in their molting. The others, numbering 79 in all, will be considered here. Of these 63 were thyroidectomized, the other 16 being incised controls. Upon comparing the results of the histological examinations with the post-operative molt periods, it was found that a thyroidectomized lizard having 7 or more follicles left in its throat shed at exactly the same intervals as the controls; but that one having 6 or less follicles left shed like completely thyroidectomized animals. Of the 63 thyroidectomized, 15 were found to have 7 or more follicles left, and 13 were found to have 6 or less follicles left. The former group we will call incompletely thyroidectomized; the latter we may group in the following account with the 35 completely thyroidectomized ones. Two apparently completely thyroidectomized lizards had molting periods like controls. In averaging these with the completely thyroidectomized group we tend to destroy the sharp difference which exists between the molting periods of the two groups. One lizard which had more than 7 follicles shed like a completely thyroidectomized lizard but was averaged with the incomplete group, and tended to break down the distinction further.

The average of the completely thyroidectomized group was 26.47 days (variation 16.33–49.0), while that of the controls was 16.43 (variation 14–20.25). The average of the incomplete group was 18.55 days (variation 14.57–21). Thyroidectomy, then, in spite of the three exceptions noted above lengthens the average molt period of *H. brookii*. In this species it does not, however, inhibit molt entirely.

Removing the thyroid of these lizards did not delay markedly the appearance of the molt immediately following the operation. In the group of 48 completely thyroidectomized lizards (including the 13 with 6 or less follicles) this molt appeared, on the average, 17.72 days after the preceding one. In the incompletely thyroidectomized series the average time was 16.3 days while in the controls it was 15.12 days. There is, therefore, a distinct lag in the effect of thyroidectomy on the molting of the lizards. Since the lag was approximately the same whether the operation was performed in the early or later part of the interval between molts, it would seem that at any one molt the tissues become so adjusted that the following molt will occur at approximately the usual time regardless of any interference with the process.

EFFECT OF THYROXIN

To establish that the delay in molt of thyroidectomized lizards was due to the absence of the thyroid and not to some secondary effect of the operation, it seemed desirable to show that the molting periodicity could be returned to normal in thyroidectomized lizards by replacement therapy. Unfortunately our series of geckos was not sufficiently large to sacrifice a number for their thyroids. We have therefore used thyroxin as a substitute. Hopping (1931) has showed that 3 days after injecting an alligator with thyroxin the tissue metabolism is raised 150 to 190 per cent above the control. Hopping used Squibbs thyroxin and we have obtained our thyroxin from the same source.

Eight lizards were selected for thyroxin treatment. Four were completely thyroidectomized, as later histological examination showed, and they were shedding at long intervals. Two were incised controls of the thyroidectomy series and 2 were normal lizards which had not been opened in the manner of the others. They received 0.3 cc. of a 1 to 10,000 solution of thyroxin on alternate days. The injections were given intramuscularly in the hind legs. After two weeks the thyroxin solution was weakened to 1 to 30,000, but the same amount was given at the same intervals.

Five other lizards were selected for the control series. Two were thyroidectomized lizards shedding in long periods. Subsequent histological check revealed that one of these controls had 2 and the other 5 follicles, but as stated above this was not sufficient thyroid tissue to keep the molting periodicity up to normal. Two other controls were stock lizards and one was an incised control. The five lizards received 0.3 cc. of salt solution (.6 per cent) at the same intervals as the experimentals.

None of the lizards stood the injecting well. In none, no matter how early in the inter-molt period the injecting was begun, was the time of appearance of the first molt following the initial injections altered materially. The next molt, however, was distinctly influenced by the thyroxin. In one thyroidectomized lizard whose molts had averaged 28 days apart, the second molt following the thyroxin treatment came 17 days after the first. Another thyroidectomized lizard with a cycle of 25.8 days shed 19 days after the first molt and then 18 days later. A third thyroidectomized lizard with a cycle of 28.2 days between molts shed in 16 and then in 18 days for the same two periods. Unfortunately, these three lizards did not live to give additional records, but it will be noted that the records obtained represent a return to the normal molting periodicity of the species. The thyroxin did not shorten the intervals between molts in the lizards having their thyroids intact. One of the incised lizards had two molts at the usual intervals and the other three molts at the same average time following the first molt after treatment. The two lizards which had not been incised died before giving any record, and the same was true of one of the thyroidectomized lizards.

The control series receiving the saline solution did not shorten the intervals between their molts. A thyroidectomized individual which has been molting on the average every 29.8 days, lengthened the period following the first molt after treatment to 32 days. An incised control shed at the usual time for the second molt. The other three controls did not live to the time for their second molt. It would seem from this record that 0.3 cc. of salt solution was too great an amount for the health of the lizards. However, the solution did not dilate the limbs greatly and we are inclined to believe that the lizards died from infections received during their frequent handling.

It seemed to us strange that thyroxin would return the delayed molt of thyroidectomized lizards to the normal and yet would not shorten the interval between molts in the unoperated controls. We therefore repeated the thyroxin experiments on other unoperated lizards but secured only similar results. CH 345 was injected 13 times with 0.25 cc. of a 1 to 3,000 solution of thyroxin over a period of 19 days. During this period it molted twice, the first time 12 days after beginning the treatment and the second time 13 days later. CH 67 received 24 injections of thyroxin, 0.25 cc. each time. Five of these injections were 1 to 10,000, 2 were 1 to 6,000, and 17 were 1 to 3,000. These injections were extended over a 34-day period. During this time the molts came at the following intervals: first at 15 days after the previous molt, then at 13 days, and finally at 15 days.

We have also attempted to change the interval between molts by implanting fresh thyroids of other species of lizards, by injecting various solutions of desiccated thyroid (Lilly) and by various solutions of adrenalin chloride. None of these treatments gave clear-cut results. In none was the average periodicity of molt distinctly increased over that of the controls.

Effect of Hypophysectomy

Removal of the anterior pituitary in Amphibia and some other vertebrates is known to inhibit the normal functioning of the thyroid. If extirpation of the thyroid delays the molting process in lizards, hypophysectomy should have a similar although less marked effect. We have, therefore, hypophysectomized a series of *H. brookii* and compared their molting records with the thyroidectomized series. The hypophysis was removed through the roof of the mouth, care being taken to avoid injury to the base of the brain. In each case we attempted to remove the entire hypophysis but were not always successful.

Twenty-six hypophysectomized lizards survived the operation and gave us 60 molting records. In this series the interval between molts dropped from a 15.73-day average to a 23.21-day average, with a range of from 14 to 39 days. Hypophysectomy, therefore, definitely lengthens the period between molts.

The heads of 10 of these 26 hypophysectomized lizards were sectioned and examined for remnants of the pituitary. It was found that one specimen, No. 57, which had the pars posterior intact, part of the pars intermedia left, and also some of the pars anterior, did not markedly lengthen its post-operative molt periods, which were 16.5, 16, 20, and 19 days. Two other specimens, Nos. 160 and 168, had only one molt period before they died and in each case it was a short one, 14 and 19 days respectively. As will be shown in other specimens, this does not necessarily mean that their subsequent periods would have been short if they had lived. Of these two, No. 160 had only a small portion of the pars posterior remaining, and No. 168 had no hypophysial tissue left. The other 7 specimens all had long periods and showed no remnant of the pars anterior.

Five of the 7 having no pars anterior left had part of the pars intermedia, part of the pars posterior left, or both. No. 124, whose post-operative periods were 19, 25, 26, and 27 days, had the pars posterior intact and portions of the intermedia left. No. 137 had some intermedia left, but no posterior, and shed in 18 and then 36 days. No. 150 had the whole posterior and intermedia left intact and shed in 25, 41, and 43 days. No. 130 had only a portion of pars posterior left but no intermedia and shed in 22, then 26 days. No. 235 had almost the whole posterior intact but no intermedia and shed in 19, 25, 31, 35, 39, 30, 29, 39, and 28 days, respectively. From the above record it is clear that either the whole or part of either the pars intermedia or pars posterior or both together are not sufficient to maintain molt periods of normal length. Pieces of the pars anterior lobe are necessary to accomplish this.

Two of the 10 hypophysectomies sectioned had no hypophysial tissue left. Number 58 shed in 14, 21, 34, 23, and 31 days. Number 134 shed in 23 days. These periods produced by lizards with no hypophysial tissue left are no longer than those produced by lizards with any amount or combinations of intermedia and posterior left. The only specimens keeping their periods within the limits of normal unoperated lizards were those having some anterior left. The pars anterior, then, influences the length of molt periods and not the pars posterior nor the pars intermedia.

The hypophysis also controls the expansion of the melanophores. It is usual for these lizards to remain quite dark under the conditions of temperature and lighting imposed by these experiments. Of the two animals, Nos. 58 and 134, which had no hypophysial tissue left, one remained very light colored from the time of operation until death. All other specimens having no pars intermedia left, *i.e.* Nos. 130, 160, 168, and 235, but which did have either some pars anterior or pars posterior left were reported as being continuously light colored. Two specimens, No. 137 and No. 57, which have some pieces of intermedia left are reported as being darker. Another, No. 124, which had some intermedia pieces, was still darker. The lizard, No. 150, which had its entire intermedia present was always dark; *i.e.* normal color.

The hypophysectomized lizards exhibited some variation in the length of time required to complete a molt once it had begun. We could not secure definite evidence that the variation was correlated with the degree of completeness of the operation. For example, one lizard in our series, No. 58, was proved by histological examination to be completely hypophysectomized, all three lobes having been removed, and yet it shed as rapidly as the controls. Another lizard, No. 168, was also completely hypophysectomized but remnants of the old shed remained attached to the skin for long periods. It is possible that a serious delay in completing the molt is correlated with some injury to the brain suffered at the time of hypophysectomy. Our sections, however, gave no evidence of such an injury.

DISCUSSION

Thyroidectomy greatly lengthens the period between molts in the gekkonid lizard, *Hemidactylus brookii*. It does not delay the rate at which the molt layer is freed from the underlying tissues and removed by the lizard. In this it stands in contrast to hypophysectomy, which both increases the period between molts and frequently, but not always, delays the process of loosening and freeing the epidermal layer to be cast. Since northern reptiles do not molt in winter, it is apparent that molting is closely correlated with the metabolic rate of reptiles. The thyroid is well known to have a marked influence on the metabolic rate of many vertebrates. The question arises whether the influence of thyroidectomy on molt is indirect, or whether thyroid function is closely bound with the molting process, as has been claimed in Amphibia.

In Amphibia at the time of metamorphosis, when it has been shown that the metabolic level is greatly increased, molting occurs for the first time in the life of the individual on a large scale. In the newt, Springer (1909) found that well-fed newts molted more frequently than less-fed controls. How greatly the metabolism of these newts was increased is not known. On the other hand, all molting in Amphibia does not appear to be correlated with increased metabolic rate. Ružička (1917) obtained an increase in the number of molts in European newts by starvation. Adolph and Collins (1925) found that gauze bandage would increase the frequency of molt in the common newt. Dawson (1920) found a molt followed drying the skin in *Necturus*. As long ago as 1894 Nicolas found that operating on a salamander induced a molt. Injury of the integument has been shown by Adolph and Collins, as well as by other workers, to call forth a molt. Obviously, excitation of the integument of Amphibia by various methods speeds up the appearance of the next molt. In Amphibia there appears to be a dual control over the mechanism of molt, one apparently hormonal and the other nervous. The endocrine and nervous systems may function vicariously in the case of the contraction of melanophores, and of smooth muscle cells. Possibly thyroid and nervous impulses to the integument both cause the molting cells to release their secretion, causing the superficial layer of the epidermis to be lifted from subjacent tissues. However, Adams and Grierson (1932) found that pilocarpine did not increase the incidence of molt in the newt, although this drug has been shown to cause release of secretion from the unicellular hatching glands of salamanders (Noble and Brady, 1930).

In reptiles the molting cells reported in Amphibia do not appear and molting is far more regular in its periodicity. In many snakes and some lizards a fluid has been found under the molted skin at the moment of shedding. This fluid has the appearance of lymph. It is possible that a similar fluid is extruded during the molting process of all reptiles, but in most lizards it dries before the old skin is actually shed. Adams and Grierson (*op. cit.*) report a fluid under the molting skin of newts which molted out of the water. They infer that this fluid is lymph and not the secretion of the unicellular hatching glands which have been described in other Amphibia. From the data at hand it is impossible to state how great a rôle the extrusion of lymph through the skin plays in the molting of either reptiles or Amphibia. Since, however, the molting of reptiles is not hastened by the various excitations reported in Amphibia, the mechanism of molt is apparently different in the two cases.

Thyroidectomy and hypophysectomy may delay the molt of reptiles merely by lowering the rate of metabolism. Molting is delayed by low temperatures and here the metabolism is obviously affected. Since molting occurs in *Hemidactylus* in the total absence of the thyroid, this gland is not an essential part of the molt mechanism.

CONCLUSION

Either thyroidectomy or hypophysectomy lengthens the period between molts in the lizard, *Hemidactylus brookii*, but neither operation entirely prevents molting. Thyroidectomy does not delay the appearance of the molt immediately following the operation but affects all later molts. If 6 or fewer follicles remain after thyroidectomy, the effect is the same as complete thyroidectomy. Injections of thyroxin, 1 to 10,000 and later 1 to 30,000 given intramuscularly to thyroidectomized lizards on alternate days, bring a return of the molt to its normal periodicity. However, neither injections of thyroxin nor implantations of fresh lizard thyroid increase the frequency of molt in the intact lizard.

Completely hypophysectomized *Hemidactylus brookii* turn a pale grey and remain this color permanently under varying environmental conditions. Lizards with fragments of the pars intermedia remaining turn a slightly dark tone under conditions where the controls are very dark grey.

Removal of the pars anterior of the pituitary alone has the same effect on delaying the molt as removal of the whole gland. The lengthening of the interval between molts may be due to a lowering of the metabolic rate of the lizard and not to any direct action of either the anterior pituitary or the thyroid on the molt mechanism.

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THE EFFECT OF ADRENALIN CHLORIDE AND TOAD VENOM ON THE BLOOD PRESSURE AND HEART RATE OF THE TROPICAL TOAD, BUFO MARINUS

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The tropical toad, *Bufo marinus*, secretes and stores in its skin glands a large amount of venom containing powerful adrenalin and digitalis-like substances without apparent harm to itself. Various workers have determined the pharmacological action and the minimum lethal dose of toad venom for animals other than the toad (Vulpian, 1854; Phisalix and Bertrand, 1893; Abel and Macht, 1912; Chen, Jensen, and Chen, 1931; Xavier, Vellard, and Vianna, 1931). Vulpian (1858) stated that the venom of the toad, which destroyed the irritability of the heart of the frog and of the salamander, had no action on the heart of the toad. Kobert (1887) found the vessels of the toad when perfused with toad venom to be less affected than those of the frog. Abel and Macht (1912) showed that both bufo-epinephrin and "bufagin," isolated from the venom, caused vasoconstriction when added to Locke solutions used to perfuse the blood vessels of *Bufo marinus*, and stated that the toad was relatively immune to "bufagin" but not to epinephrin. In the South African clawed toad, *Xenopus lævis*, Gunn (1930) found an adrenalin-like substance in the skin secretion which produced striking circulatory effects in the cat and the rabbit, but neither the skin secretion nor adrenalin had an effect on the circulatory system of *X. lævis*.

No reference in the literature could be found concerning the effect of adrenalin chloride or of toad venom on the blood pressure of the toad, nor even in any way concerning the measurement of blood pressure in this amphibian. Bieter and Scott (1929) found that an intravenous injection of 0.2 cc. of adrenalin chloride, 1 : 10,000, gave a rise of blood pressure lasting one hour and a quarter in the frog, *Rana pipiens*. The present report concerns the effect of intravenous injections of adrenalin chloride and of toad venom on blood pressure and heart rate in *B. marinus*. A determination of the minimum amounts of these substances necessary to produce a rise in blood pressure on intravenous injection is also reported.

METHOD

Specimens of *B. marinus* weighing from 215 grams to 590 grams were prepared by destroying the fore-brain through the *os fronto-parietale* and pithing the spinal cord posterior to the second vertebra. The blood pressure was recorded by a mercury manometer of 3.5 mm. bore from a cannula in the *A. femoralis*. Sodium citrate, 7 per cent, was used as an anticoagulant and the cannula was wet with heparin before insertion. Injections were made with a Luer tuberculin syringe through a cannula in the *V. femoralis*. For threshold determinations the volume of fluid was generally not more than 0.2 cc. An intravenous injection of 0.5 cc. of amphibian Ringer's solution was without effect. Adrenalin chloride (Parke, Davis and Co.) was used in dilutions with Ringer's solution from 1 : 50,000 to 1 : 50,000,000. The venom was expressed on a weighed cover-glass from the large gland behind the ear, weighed, dissolved in Ringer's solution to known dilutions, and injected immediately. Laboratory temperatures varied from day to day between 27° C. and 29° C.

RESULTS

The average systolic blood pressure in the femoral artery in nineteen toads about one-half hour after the destruction of the cerebral hemispheres and spinal cord and before injection was 33 mm. Hg, ranging from 22 mm. Hg to 56 mm. Hg. The heart rate was 76 per minute and the respiration 106 per minute. The pulse pressure varied from 4 mm. Hg to 12 mm. Hg. In two toads under ether anesthesia, with the nervous system intact, the blood pressure was 32 mm. Hg, the heart rate 64, and the respiration 106.

In twenty-four toads an intravenous injection of 0.2 cc. of adrenalin chloride 1 : 50,000, which was 0.8 μg. to 2 μg. per 100 grams (1 μg. equals 0.001 mg.), produced a rise in systolic blood pressure of 21 per cent to 155 per cent which lasted from 2 to 6 minutes (Fig. 1, *A*). Weaker doses such as 0.2 cc. of 1 : 200,000 regularly produced a pressor response; thus in one instance this dose was 0.33 μg. per 100 grams and gave a rise of 56 per cent. Subsequent doses were just as effective as first doses. The minimal effective dose of adrenalin chloride for a pressor response was found in nine toads to average 0.05 μg. per 100 grams with a range from 0.02 μg. to 0.09 μg. No depressor responses occurred even with doses as low as 0.002 μg.

While the effect of adrenalin on the heart rate in these preparations was not uniform but varied with the strength of dose and condition of the animal, there was generally a decrease. In fifteen toads doses from 0.2 cc. of 1 : 200,000 to 0.5 cc. of 1 : 50,000 (0.33 μg. to

2.5 μg. per 100 grams) gave a fall in heart rate of 6 per cent to 67 per cent associated with the rise of blood pressure. In three cases a rise of heart rate of less than 10 per cent occurred with a relatively large dose (1 μg. per 100 grams). In one case with a low heart rate of 44 per minute a dose of 2 μg. caused an increase to 56 per minute, while twenty minutes later a dose of 2.5 μg. caused a fall in heart rate from 68 to 57.

An intravenous injection of toad venom in Ringer's solution produced a rise in systolic pressure in ten toads in all doses from 160 μg. to 4 μg. somewhat in proportion to the amount of venom (Fig. 1, *B*). Thus, for example, 160 μg. gave a 292 per cent rise, while 15 μg. and 4 μg. gave 83 per cent and 33 per cent respectively. The minimum effective dose for a pressor response was found in five toads to average 0.5 μg. per 100 grams, with a range from 0.32 μg. to 0.71 μg. No depressor responses occurred with the small doses.

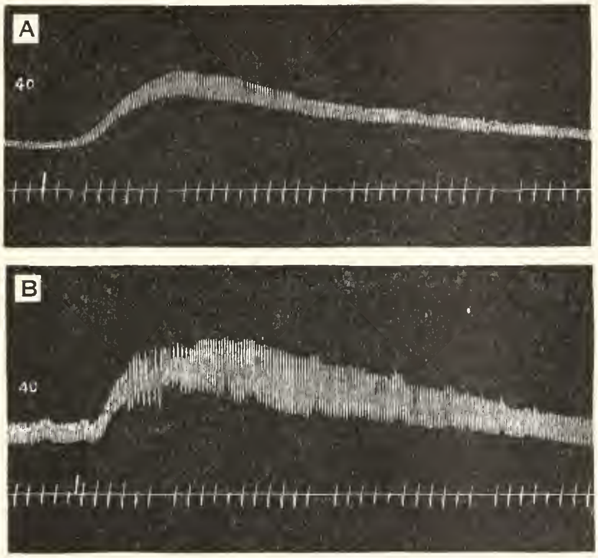

FIG. 1. *Bufo marinus*, 450 gram ♀, 29° C. Effect of intravenous injections on blood pressure and heart rate. *A*. Adrenalin chloride, 0.9 μg. per 100 grams. Rise in blood pressure, 145 per cent. Fall in heart rate, 9 per cent. Minimal pressor dose, 0.04 μg. *B*. Toad venom, 71 μg. per 100 grams. Rise in blood pressure, 123 per cent. Fall in heart rate, 22 per cent. Minimal pressor dose, 0.7 μg. Time in five-second intervals.

In nine toads the intravenous injection of the diluted toad venom caused a decrease (maximum 40 per cent) in the heart rate associated with the rise in pressure. In one case an increase occurred both with the venom and with adrenalin.

DISCUSSION

The cardio-inhibitory effect of adrenalin associated with a rise of blood pressure suggests the presence of a reflex mechanism similar to that in mammals and certain lower vertebrates since in both the elasmobranch, *Squalus acanthias*, and the amphibian, *Necturus maculosus*, Lutz and Wyman (1932*a*, 1932*b*) have shown a reflex cardio-inhibitory mechanism stimulated by increased intravascular pressure.

In the reflex toad preparation the cardio-inhibition following the injection of venom must be due partly to the direct effect of "bufagin" (Abel and Macht, 1912) or cinobufotenine (Chen, Jensen, and Chen, 1931), although there was nothing in the response either of the blood pressure or of the heart to distinguish the effect of the venom from that of adrenalin alone.

Dragstedt, Wightman, and Huffman (1928) found the minimal effective pressor dose of adrenalin in the unanaesthetized dog to be less than from 0.2 cc. to 0.4 cc. of 1 to 1 million per kilogram per minute (0.02 μg. to 0.04 μg. per 100 grams). In rats, under urethane, Wyman and tum Suden (1932) generally obtained a detectable pressor response to adrenalin with 0.02 μg. per 100 grams, but in normal rats they found that 0.04 μg. per 100 grams was necessary to produce a visible vasoconstriction of the mesenteric vessels. These figures when compared with the pressor threshold for *B. marinus* to adrenalin (0.02 μg. to 0.09 μg. per 100 grams) indicate that this amphibian and the mammal are about equally sensitive to adrenalin. The total amount present at any time in the skin glands of the toad, the secretion of which yields about 5 per cent adrenalin according to Abel and Macht (1912), must be a great many times that present in the adrenal of the mammal (Sollman, 1932). According to Chen, Chen, and Jensen (1932) the adrenalin of the skin glands does not enter the blood stream although Phisalix and Bertrand (1893), using a questionable procedure, believed they found the toad venom in the blood in sufficient quantity for biological assay.

Since no depressor responses were obtained with adrenalin in doses as low as 0.002 μg. (one twenty-fifth the average pressor threshold), it appears that there are no vasodilators present in *B. marinus* sensitive to adrenalin.

SUMMARY

1. Adrenalin chloride injected intravenously in *Bufo marinus* in doses of 0.33 μg. to 2 μg. per 100 grams produced a rise in systolic blood pressure of 21 per cent to 136 per cent. The average minimal effective dose was 0.05 μg. per 100 grams, ranging from 0.02 μg. to 0.09 μg.

2. Doses of 0.33 μg. to 2.5 μg. per 100 grams generally caused a decrease in heart rate of 6 per cent to 67 per cent associated with the rise of blood pressure.

3. Toad venom injected intravenously in *Bufo marinus* in doses of 4 μg. to 160 μg. per 100 grams produced a rise of systolic blood pressure of 33 per cent to 292 per cent. The average minimal effective dose was 0.5 μg. per 100 grams, ranging from 0.32 μg. to 0.71 μg.

4. The vasoconstrictor mechanism of *Bufo marinus* is one-tenth as sensitive to its venom as to adrenalin.

5. The response to adrenalin in *Bufo marinus* suggests the existence of a reflex cardio-inhibitory mechanism stimulated by increased intravascular pressure

6. No vasodilators are present in *Bufo marinus* sensitive to adrenalin chloride in doses down to one twenty-fifth of the minimal effective pressor dose.

7. The vasoconstrictor mechanisms of *Bufo marinus*, of the dog, and of the rat are about equally sensitive to adrenalin.

I wish to thank Dr. J. F. G. Wheeler, Director of the Bermuda Biological Station for Research, for placing the facilities of the station at my disposal.

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THE STRUCTURE OF EPIDERMAL MELANOPHORES IN FROG TADPOLES

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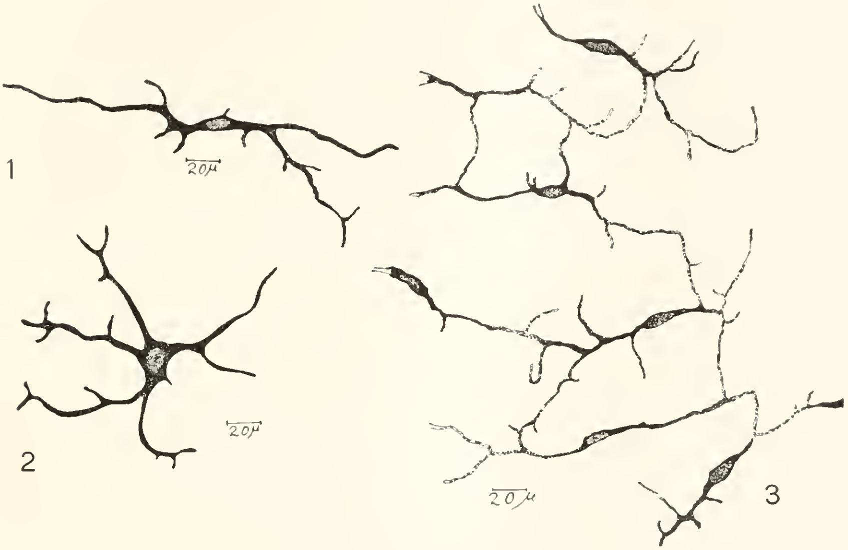
Color changes in animals have been the object of much investigation. Parker (1930), in an historical account of the subject, indicates that color changes have been recognized from very early times and are still a fertile field for study. Many problems concerning the reactions of pigment cells have been worked out. The structure of the pigmentary elements, however, has been difficult to determine. The main currents of opinion concerning the structure and the mechanism of "expansion" and "contraction" of the melanophores may be ascertained from recent papers by Hewer (1923), Matthews (1931), and Perkins and Snook (1932).

Hewer states that no fewer than one hundred and fifty papers have been published on color changes of amphibians alone. Even with this extensive work on amphibians little mention is made of the *epidermal* melanophores of frogs and their tadpoles.

In the frogs and tadpoles that I have studied, *Rana clamitans*, *R. catesbeiana*, *R. sylvatica*, *R. pipiens*, and one species of *Hyla*, two types of melanophores are present in the skin. Those of the dermal type as described by Hewer are very similar to the dermal melanophores of other vertebrates. In contrast to the dermal type, the melanophores found in the epidermis are greatly elongated and usually possess few branches (Figs. 1, 2, and 5). The usual length of single epidermal melanophores is from 150 to 200 μ. One cell 246 μ long was found but this may not be the greatest length to which they occur. It is at times difficult to ascertain the exact length of certain melanophores because of anastomosing of branches. Anastomosing of branches is rather common in regions where the melanophores are close together (Fig. 3). In about the middle of each epidermal melanophore there is a widened region which the writer has called the "cell body." The cell body is usually 15 to 18 μ in length and 7 to 10 μ wide. The branches average about 3 μ in width. The ellipsoidal nucleus lies within the cell body. The melanophores are not located at any particular level of the epidermis but they have never been observed in the cuticular layer of cells (Fig. 6). One part of a melanophore may be at a different level in the epidermis from that in which another part lies.

Observations on fully expanded melanophores in preserved or freshly excised skin are fairly easily made and the outlines of the cells may be studied in detail. This method of study, however, does not reveal, necessarily, the exact structure of the melanophore. A melanophore as seen in the "contracted" phase contains all or nearly all of the pigment within the cell body (Figs. 4 and 7), giving it an appearance strikingly different from that in the "expanded" phase in which the pigment is distributed throughout the branches (Figs. 1 and 5). Immediately the question is raised as to the nature of the change from one phase to the other.



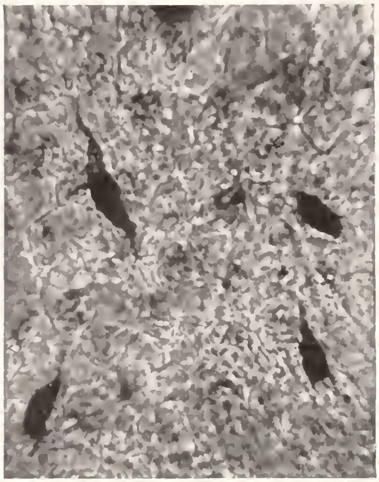
Explanation of Plate I

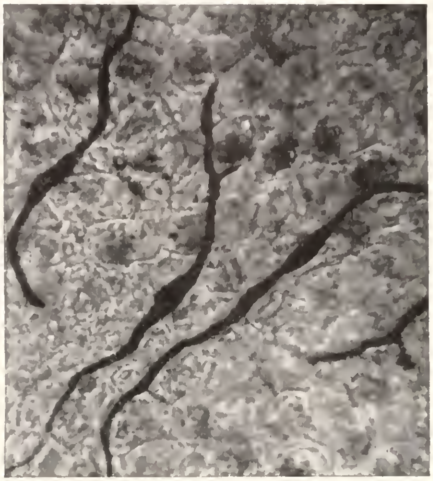
Figs. 1 and 2. Camera lucida drawing of expanded epidermal melanophore of frog tadpole. From living, uninjured tissue.

Fig. 3. Camera lucida drawing of partly expanded epidermal melanophores showing anatomosis of processes. From living, uninjured tissue.

Hewer (1923) and Matthews (1931) have described melanophore change as a migration of the pigment granules within permanent processes. Their conclusions were that the processes did not change in size or shape, the granules of pigment migrating through the semi-fluid protoplasm of the processes. These conclusions were drawn from studies on the dermal melanophores of the frog and those of *Fundulus*. There is considerable evidence that the same condition exists in the epidermal melanophores of tadpoles.

In order to study melanophores in undamaged tissue, the method used was that described by Herrick (1932) in studying migrating epidermal tissue. By this method magnifications up to nearly 1000 diameters were used successfully with the animal under only light anesthesia. Observations in some instances were made on a single melanophore over a period of several hours. The difficulty investigators have encountered has been to observe in living tissue the processes of the melanophores when devoid of pigment. The walls of the processes are very thin and have about the same refraction as the surrounding tissue. With the aid of the apparatus I used, however,





Explanation of Plate II

Fig. 4. Photomicrograph of contracted epidermal melanophores of frog tadpole. Living, uninjured tissue.

Fig. 5. Photomicrograph of expanded epidermal melanophores of frog tadpole. Living, uninjured tissue.

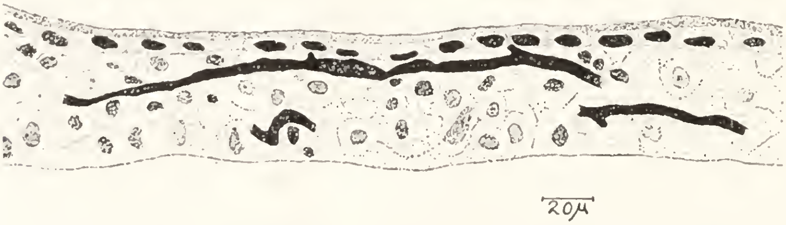


Fig. 6. Camera lucida drawing of section of tadpole epidermis showing positions of epidermal melanophores. Only fragments of melanophores are seen.

it was possible to distinguish clearly the processes of the epidermal melanophores when devoid (contracted phase) of their pigment (Figs. 8, 9, and 10). This observation proves that the processes are not temporary and that contraction is not dependent upon their reduction in size. Hewer considered it very significant evidence, in support of his conclusion that the processes were permanent, when he found scattered pigment granules some distance from the cell body during the contracted phase. This condition was found repeatedly in the tadpole (Figs. 9 and 10). These single granules moved about slightly as is usually the case with pigment granules wherever they are found.

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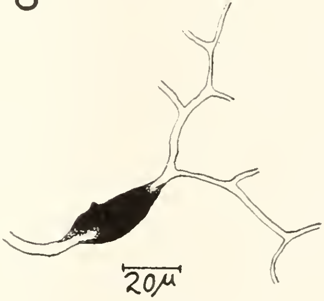

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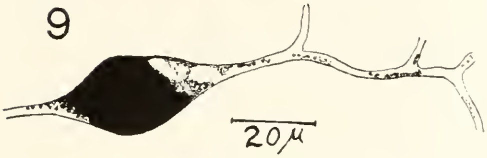

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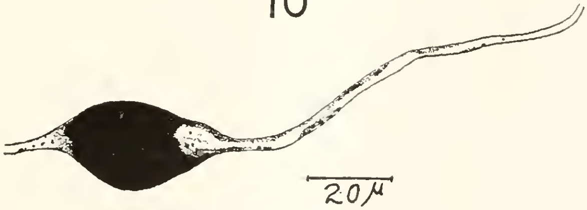

Explanation of Plate III

Fig. 7. Camera lucida drawing of cell body of contracted epidermal melanophore showing nearly all of the pigment contained within the cell body. From living, uninjured tissue.

Fig. 8. Camera lucida drawing of contracted epidermal melanophore of frog tadpole showing processes devoid of pigment. From living, uninjured tissue.

Figs. 9 and 10. Camera lucida drawing of nearly contracted epidermal melanophore of frog tadpole showing scattered pigment granules in the processes. From living, uninjured tissue.

Expansion of the melanophore is a process of dispersion of the pigment granules which had been congregated within the cell body. The length of time required for melanophores to expand maximally depends upon the nature and the intensity of the stimulus. The epidermal melanophores of large tadpoles, after intraperitoneal injection of 0.2 cc. of obstetrical pituitrin, expanded maximally in about 30 minutes. Animals placed in small aquaria, the sides and bottom of which had been covered with dead-black paper, became adapted to the dark background (almost maximal expansion of the melanophores) in from one to three hours or even longer. The individual pigment granules move so slowly that motion is barely perceptible under the microscope. There was never any suggestion of Brownian movement, a finding which is in contrast with the observations of Perkins and Snook (1932) in crustaceans. The rate of movement of a single granule was not uniform over a period of minutes and often differed from that of other granules in the same general area. The pigment most remote from the cell body was not massed together; instead, the granules were much scattered, in no wise suggesting a pseudopodium-like process being thrust out.

Contraction was a reversal of the process of expansion. During contraction the distal pigment was considerably scattered, with individual granules trailing far behind the main mass. With the employment of an ocular micrometer, no difference in the diameter of the cell body could be ascertained in the expanded and contracted phases.

CONCLUSIONS

1. Anastomosing of processes of epidermal melanophores in frog tadpoles is common.

2. The usual length of epidermal melanophores is from 150 to 200 μ.

3. "Expansion" and "contraction" are accomplished by the migration of pigment granules into and out of permanent processes.

4. Melanophore processes devoid of pigment were observed in living, undamaged tissue.

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THE INTRACELLULAR CANALICULI OF THE PHARYNGEAL GLANDS OF THE HONEYBEE[1]

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Recent studies of the intracellular secretory canaliculi in the salivary glands of the grasshopper and in the parietal cells of the stomach (Beams and King, 1932*a* and *b*) have reëmphasized the presence of these remarkable structures in certain types of gland cells. It is indeed surprising that they have received so little attention in current cytological literature unless perhaps due to an inherent skepticism derived from Holmgren's mistaken conception of them. The evidence seems decisive that these structures, together with the secretory material in the salivary glands of *Chironomus* larvæ (Krjukowa, 1929; Beams and Goldsmith, 1930; and Beams and King, unpublished), have been erroneously interpreted by Parat and Painlevé as Golgi apparatus or "vacuome." This unfortunate oversight on the part of Parat and his associate has led them to introduce on an unsound basis a new conception regarding the significance of these peculiar structures.

The pharyngeal glands of the honeybee offer exceptionally favorable material, not only for the study of intracellular secretory canaliculi, Golgi bodies, mitochondria, and the formation of secretion globules, but also for a study of the topographical relationship between these cytoplasmic components and secretory inclusions. Accordingly, this study was undertaken in an effort to advance the analysis of the question and significance of the intracellular canaliculi, Golgi bodies, mitochondria, and their relationship, if any, to the formation and the elimination of the secretory material by the cell.

Material and Methods

The material used in this study consisted of the pharyngeal glands of the worker honeybee. The head of the bee was placed on a glass slide in a drop of normal saline under the dissecting microscope and a median incision was made, dividing the head into two parts. By use of dissecting needles the glands were removed from the anterior lateral region of the head cavity and if whole mounts of the entire gland were desired, which incidentally is the best and simplest way to demonstrate the intracellular canaliculi, the remains of the head were removed from the area surrounding the glands and a cover glass applied. This whole operation takes less than two minutes and offers the clearest demonstration of intracellular canaliculi in living material of any tissue that we have as yet encountered. In fact, it offers splendid material for demonstration purposes for classes in cellular anatomy.

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[1] Part of this work was done at the Marine Biological Laboratory during the summer of 1932.

Permanent whole mounts of the glands were made by fixing them in Bouin's solution followed by staining in Delafield's or Heidenhain's hematoxylin. These methods were also very useful in the study of sectioned material. For a demonstration of Golgi bodies Nassonov's method proved successful. The mitochondria were revealed by the method of Regaud.

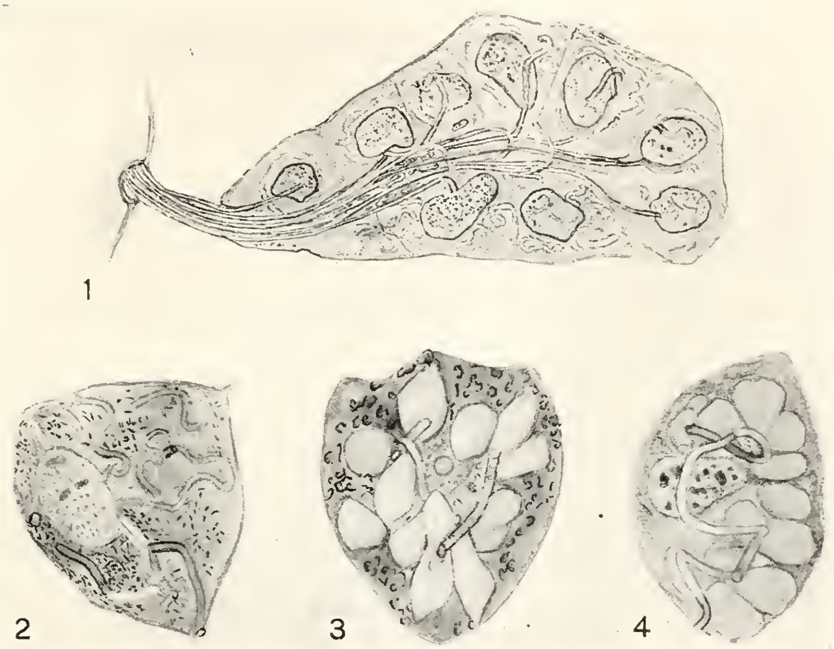
DESCRIPTION

The pharyngeal glands of the worker bee are closely packed about the brain in the upper lateral and anterior part of the head cavity. Each gland consists of a rather long common chitinized duct to which are attached numerous pear-shaped lobules composed of groups of ten to twenty cells. Occasionally lobules consisting of a single cell are found. The lobules extend in all directions from the common duct and each is surrounded by a thin, transparent, yet somewhat tough, membranous capsule which extends as a closed sac from the common duct (Fig. 1).

The cells of the lobules are closely packed together with their bases in direct contact with the enveloping tunica propria. Unlike the lobules of most glands there is present here no lumen, but a central core which contains the chitinous intercellular efferent tubules which lead singly from each cell to the common chitinized duct. Proximally the number of tubules in the core increases as others emerge from the cells of the middle and basal portion of the lobule. Thus the length of a tubule varies with the position of the cell it serves.

These efferent tubules have their source within the cell as simple somewhat sacculated canaliculi without chitinous linings; they take within the cells a rather lengthened sinuous course, coil about the nucleus, and end blindly in the cytosome. Due to this location the ducts are in direct contact with the cytoplasm and when large secretion masses are present they pass through and between them. As the intracellular ducts emerge from the cells each possesses a chitinous lining and finally passes parallel with the others through the enveloping membranous tube formed by the capsule of the lobule (Fig. 1). As the ductules reach the common duct they penetrate it by independent perforations giving rise to papilla-like elevations having, according to Cheshire (1886), a sieve-like end. Upon careful examination the small nuclei of hypodermal cells, which secrete the chitinous lining, can frequently be observed here and there along the efferent tubules. This chitinous lining apparently serves to keep the delicate tubules open without making them unnecessarily thick and heavy.

Plate I



1. General topography of a single lobule from the pharyngeal gland of the honeybee. Cells with associated intra- and intercellular canaliculi which lead to the common duct. Whole mount, Bouin, iron hematoxylin.

2. Single cell from pharyngeal gland of honeybee. Intracellular canal and mitochondria. Regaud, iron hematoxylin.

3. Single cell from pharyngeal gland of honeybee. Intracellular canal, secretion globules, and Golgi bodies. Nassonov.

4. Single cell from pharyngeal gland of honeybee. Intracellular canal surrounded by secretion globules. Canal reconstructed from adjacent sections. Champy, iron hematoxylin.

Small globules of secretion are sometimes present in the cells of newly emerged bees. When little secretion is present the globules have a tendency to be located about the nucleus; however, at the end of the secretory cycle they become so large as to mask the cytoplasm almost completely. This secretory cycle has been studied in some detail by Kratky (1931). In such cells the intracellular secretory canaliculi wind back and forth among and, in some cases, even through the secretion bodies (Figs. 3 and 4). Thus, secretion may pass directly into the canaliculi without traversing great areas of cytoplasm, which would present an effective bar to substances of low diffusibility.

The Golgi material is present in the cells of the pharyngeal gland in its typical insect form, *i.e.*, Golgi bodies with an outer osmiophilic rim or crescent associated with an inner, lighter, and less distinct osmiophobic area (Fig. 3). These are, in general, evenly distributed throughout the cytoplasm and show no indications whatsoever of having any regular morphological relationship to the developing secretory globules. However, in cells packed with secretion, the Golgi bodies are concentrated in the cytoplasm and may, of course, be seen in close but probably fortuitous association with the secretory globules.

The mitochondria have the form of short rods and filaments and, like the Golgi bodies, are distributed fairly regularly throughout the cytoplasm (Fig. 2). Here also there is no morphological evidence of any direct part played by the mitochondria in secretion as has been held by some. The mitochondria are quite distinct from the Golgi bodies and show no signs of transformation from a "passive" to an "active" condition.

Recently, much discussion has arisen regarding the synthesis of secretory products by Golgi material. In fact, the extensive communications of Nassonov and Bowen seem to leave little doubt that the Golgi apparatus is the sovereign structure concerned in the production of secretion. While the contributions of Nassonov, Bowen, and others represent the most careful application of modern cytological technique to secretion, we are not yet convinced that the Golgi apparatus plays an exclusive rôle in the synthesis of all these products. As we have seen from the foregoing description, the Golgi bodies do not in this material show a close topographical relationship to the developing secretory inclusions which would be necessary to establish a direct physiological relationship. To be sure, in many instances a Golgi body is found in direct contact with a developing secretory inclusion, but this, it seems to us, may very likely represent simply a chance position. Furthermore, no great changes in volume of Golgi bodies during the secretory phase are noticeable as might be expected if they directly functioned in the secretion of such enormous globules.

In addition, evidence has been presented by Beams and King (1932*a*) that the limiting membranes of the contractile vacuoles and their associated canals do not represent Golgi material contrary to the views of Nassonov (1923, 1924, and 1925). Nassonov was led to his conception by the fact that these limiting membranes in the contractile vacuole of certain ciliates blacken in osmic acid and are, according to him, involved in secretory activity. Beams and King have also shown that the limiting membranes of intracellular canals in the grasshopper material studied by them usually blacken in osmic acid, while there is present the usual insect Golgi material. The intracellular canals of the pharyngeal glands of the honeybee do not reduce osmic acid just as in certain ciliates the walls of the contractile vacuole do not. This blackening of structures by osmic acid may be held, then, to be due to their chemical nature rather than to their homology with each other.

Conclusions

1. The pharyngeal glands of the honeybee are composed of lobules which contain ten to twenty unicellular glands.

2. Each unicellular gland possesses an intracellular secretory canaliculus, readily demonstrable in the living condition, which becomes continuous at the apex of the cell with an intercellular chitin-lined duct which joins the common duct of the gland.

3. No evidence was observed to support the theory that the Golgi bodies synthesize the secretory material.

4. In like manner no evidence was observed that the mitochondria directly function in secretion.

5. It is clear that the mitochondria and the Golgi bodies are discrete in the cells of the pharyngeal glands of the honeybee.

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COLOR CHANGES IN FUNDULUS AFTER HYPOPHYSECTOMY

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The pituitary gland is of primary importance in amphibian color change (Hogben, 1924) and its removal from the dogfish produces effects which are similar to those observed in the hypophysectomized frog (Lundstrom and Bard, 1932). In teleosts, however, evidence for any influence of this gland on color change is inconclusive. Certainly in *Fundulus* the activities of the melanophores, and probably of the xanthophores as well (Fries, 1931), are controlled mainly by the nervous system. Nevertheless, recent experiments have shown that other factors, probably humoral in nature, exert some control over the denervated melanophores (Smith, 1931; Parker, 1932) and xanthophores (Fries, 1931), enabling them to respond in an appropriate manner to changes in background though much more slowly than do those pigment cells which remain connected with the nervous system. As Smith points out, these factors may play some part in the activities of innervated pigment cells as well, "probably—furthering the action of the nervous system when the animal remains on the same background for a relatively long period." The nature of the hormone which produces these effects, however, is in doubt. Parker believes that neurohumoral substances are responsible for the changes in tint of denervated areas in *Fundulus.* Smith states that "while in Phoxinus at least humoral factors exert an influence upon the behavior of the melanophores it is at the present moment impossible to enlarge upon this statement," but he suggests the possibility of adrenalin and pituitrin playing a part. Certainly extracts of the posterior lobe of the pituitary do produce definite changes in the pigment cells of both *Fundulus* and *Phoxinus.* Spaeth (1918) found that pituitrin produced a concentration of melanophore pigment when isolated scales of *Fundulus* were immersed in it, and Wyman (1924) obtained similar results with injections of this drug. Hewer (1926) also described a concentration of melanophore pigment and in addition a dispersion of xanthophore pigment following the injection of pituitrin into a minnow, although he is of the opinion that the pituitary gland plays no part in normal color change since the amount of pituitrin necessary to bring about these changes is, he believes, too large to be produced by the animal itself. In *Phoxinus* the effects of injections of pituitary extracts are apparently different from those observed in *Fundulus*. Abolin (1925) obtained in this fish a dispersion of melanophore, xanthophore, and erythrophore pigments following an injection of infundin and he believed the injection acted in the same way as does the normal reaction to a black background. Similarly, as a result of an injection of infundin, Giersburg (1930) observed a definite dispersion of pigment granules or "expansion" of the melanophores on the sides of *Phoxinus*, together with a pronounced "expansion" of the xanthophores and erythrophores, although he did obtain a "contraction" of the melanophores on the back of this fish. He suggests that the xanthophores and erythrophores and possibly a few melanophores (*e.g.* those on the distal parts of the fins) are controlled by humoral factors, "expansion" of these cells being a result of pituitary activity.

Recently Zondek and Krohn (1932, *a* and *b*) have isolated a substance which they call *intermedin*, from the intermediate lobe of the pituitary of various animals including *Phoxinus*, which, when injected into this fish, produces an "expansion" of the melanophores, xanthophores, and erythrophores. These authors claim that this substance is the only one which will produce this expansion of the erythrophores and that duplication of the red coloration which this fish exhibits during the breeding season can be obtained by injecting a suitable amount of intermedin.

It would appear, then, that humoral factors probably play some part in the color changes of teleosts. Moreover, since the injection of pituitary extracts produces definite changes in color in these fishes, the pituitary gland may be suspected of taking some part in this humoral influence on color change, particularly since this gland is of such importance in color changes of amphibians and its removal has such a pronounced effect on the color pattern of the dogfish. In order to determine what rôle, if any, is played by the pituitary gland in color changes in *Fundulus* the following experiments were carried out.

The hypophysis was removed from a number of killifish (*Fundulus heteroclitus*) in the following way. Each fish was fastened on its back by two strips of wet cloth that were passed across its body and secured to a piece of cork with thumb tacks. A V-shaped cut through the branchiostegal membrane was made with the base of the V at the tip of the tongue. The tongue was then pulled ventrally through the incision which was extended dorso-laterally far enough to expose the region of the hypophysis in the roof of the oral cavity. Usually the hypophysis itself could be seen through the mucous membrane and bone. The mucous membrane was now cut longitudinally a little to one side of the mid-line (to avoid a median artery) and the tissues loosened from the bone. At first a dental burr was used to penetrate the bone ventral to the hypophysis but this approach left the field so clouded with bone dust that it was difficult to be certain when the hypophysis was completely removed. To avoid this in the majority of cases the bone was cut through with iridectomy scissors and a small window removed, thus exposing the hypophysis which could then be drawn out with forceps or with a fine pipette. The *circulus cephalicus* was frequently cut on one side, but the animals recovered from these operations about as readily as when none of the main arteries had been cut. After the operation no attempt was made to close the incision in the floor of the mouth. The animals were merely kept in N/10 sodium chloride for from 24 to 48 hours and then removed to tap water. In all cases the individuals were examined after death to determine whether or not the hypophysis had been completely removed. Those cases where part of the gland was left behind served as controls. Additional control animals were obtained by performing a number of dummy operations in which the hypophysis was exposed but left intact.

Animals hypophysectomized in this way lived for several weeks, during which time their reactions to background changes were examined. As usual a number failed to react, a common experience even with unoperated animals. The majority of both the hypophysectomized and control animals, however, reacted exactly as do normal animals when placed over white, black, or yellow backgrounds, becoming light over white, dark over black, and a decided yellow color over the yellow background. Microscopic examination of the tail fins of these animals showed that, as usual, the melanophores were contracted over white and yellow backgrounds and expanded over black, while the xanthophores were contracted over white and expanded over yellow. Many of the animals became lighter in color a few days after the operation due to degeneration of some of the melanophores, as could be seen when a scale or the tail fin was observed under the microscope. But here again no difference could be detected between hypophysectomized and control animals, hence the bleaching of these animals could not have been due to the absence of the hypophysis.

Parker and Lanchner (1922) have shown that when a *Fundulus* is put in total darkness it becomes light due to contraction of its melanophores. This reaction was also tested. When a light-proof box was placed over the aquarium in which a hypophysectomized animal was swimming, the removal of this hood an hour or so later showed that the fish had become definitely lighter in color, again behaving like a normal animal.

In order to determine whether or not the pituitary body is concerned with the slow changes in tint of denervated areas which Smith (1931) and Parker (1932) have observed, the following experiments were performed. A transverse cut was made in the base of the tail of six animals following the technique described by Fries (1931). A black area extending distal to the cut gave evidence that the nerves running to the melanophores in this area had been severed. The hypophyses were then removed from three of these animals, the other three serving as controls. When kept continuously over a white background it was found that the denervated melanophores of the tail contracted at approximately the same time in both hypophysectomized and control animals, about three days being required to complete the change in both cases. Similar results were obtained for denervated areas of the trunk. Although the reaction here was not as definite as that in the tail, nevertheless black denervated areas of the trunk did become smaller after several days over a white background in hypophysectomized as well as in control animals. Obviously removal of the hypophysis does not prevent denervated areas from changing in tint when the fish are transferred from one background to another.

It is apparent from these experiments that the removal of the hypophysis from *Fundulus* does not affect the ordinary reactions which it exhibits to changes in background or to total darkness. But since commercial posterior lobe extracts do cause the melanophores of this fish to contract (Spaeth, 1918; Wyman, 1924), the question naturally arises as to whether or not the pituitary body of *Fundulus* contains any of the melanophore principle. The pituitary bodies of other fish have been shown to contain this principle. Hogben and Winton (1922) found that extracts of cod pituitary produced the usual effects when injected into frogs, and Zondek and Krohn (1932*b*) have shown that "intermedin" can be extracted from the pituitary of *Phoxinus*. The following experiment shows that the hypophysis of *Fundulus* also contains the melanophore principle. The pituitary, forebrain, medulla, and a small piece of trunk muscle were removed from a *Fundulus* of average size (6 to 8 centimeters in length) and separate extracts were made of each by grinding them in 0.05 cc. of N/10 sodium chloride solution. As test objects a number of scales from another fish were mounted in hanging drops of the same medium in which, as usual, the xanthophores contracted and the melanophores expanded. When the fluid on one of these scales was exchanged for the brain or muscle extracts no change was observed in the pigment cells In fact, when scales with contracted melanophores and expanded xanthophores were mounted in these brain extracts, the melanophores would expand and the xanthophores contract, the brain extract thus having the same effect that ordinary sodium chloride solutions would have. But when a scale preparation was changed to the pituitary extract, the xanthophores always expanded, while the melanophores contracted. Obviously these extracts of the pituitary body were crude and probably only a small proportion of the active principle or principles of the posterior lobe could be removed in this way (Kamm et al., 1928). As a matter of fact, the reaction of the pigment cells was slower than that produced by N/10 KCl, for example, as several minutes were required for the maximum effect to be reached and even then the melanophores were not always contracted to a punctate condition. Moreover, the effect of the pituitary extract lasted for only about 45–60 minutes, after which the melanophores slowly reëxpanded, and although they could be made to contract again by adding the extract a second time, this second contraction was rarely as complete as the first. Nevertheless, these experiments show that the pituitary body of *Fundulus* does contain a substance which will produce an expansion of the xanthophores and a contraction of melanophores in isolated scale preparations. How this fact is to be reconciled with the results described above, namely that the removal of this gland has no apparent effect on subsequent color changes of the animal, is not evident from these experiments. It is possible, as Hewer (1926) suggests, that any melanophore principle formed by the hypophysis is liberated in quantities too small to have any effect on the pigment cells. However this may be, it is clear from the experiments first described that the removal of the hypophysis from a *Fundulus* does not affect the ability of that animal to change its color in apparently the same manner as does a normal animal.

Summary

When the hypophysis of a *Fundulus* is ground up in 0.05 cc. N/10 NaCl and a scale from this fish is mounted in such an extract, the xanthophores "expand" while the melanophores "contract," although brain and muscle extracts prepared in the same way have no effect on the pigment cells. The hypophysis of *Fundulus* does contain the melanophore principle. In spite of this fact, when the hypophysis is removed from *Fundulus*, the animal still responds to changes in background and to total darkness in the usual manner. Moreover, the slow change in tint of denervated areas occurs as readily in hypophysectomized as in normal animals. In other words, the removal of the

hypophysis has no apparent effect on the pigmentary responses which *Fundulus* may exhibit.

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A NOTE ON AMPHIOXIDES FROM BERMUDA BASED ON DR. W. BEEBE'S COLLECTIONS

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The "Challenger" Expedition caught on the high seas a peculiar pelagic branchiostomid to which later the name of *Amphioxides* was given by Gill. Our knowledge of these forms dates from the author's monograph (1905) based on the material of the "Valdivia" Expedition. The interesting anatomy was thoroughly studied, resulting in the discovery of whole organ-systems not yet known in the Acrania, viz. the visceral musculature, the musculature of the mouth, the canal-system of the head region, connective tissue and skeletal system, the nephridial nature of the so-called Hatschek's nephridium, discoveries which have since all been verified for *Amphioxus*. The question which then arose was whether these *Amphioxides*-forms represented a family of pelagic Acrania or whether they were peculiar larval forms. In his first monograph the author decided in favor of the first view, because all *Amphioxides* were caught far away from the shore and mostly in considerable depth; also because up to 35 gill-slits specimens were found without a trace of metamorphosis; and finally because some of the largest specimens had already nicely developed gonads. Of course the possibility was discussed that larvæ of some species of Acrania were carried out to the high seas and became there neotenic. Only one year later (1906) the author had to accept the latter alternative because he received from the North Seas a larva of the ordinary *Branchiostoma lanceolatum* which had not undergone metamorphosis at the usual stage but grown into an *Amphioxides* larva. A few years later (1909) the author was able to study still other material which yielded further information. The new material did not come from the deep sea but from the surface. The decisive fact was the presence of a number of individuals, taken off the mouth of the Amazon river and belonging to the species *A. valdiviae* Goldsch. which had begun with metamorphosis at stages with 25–34 gill-slits. This find, of course, settled the larval character of the *Amphioxides* forms. In 1910 appeared a paper by Gibson on the basis of the material of the Percy Sladen Trust Expedition, which had collected the largest amount of *Amphioxides* material yet known. Besides a few minor discrepancies in details of anatomy, some of which have since come out in favor of my description, the author adds the fact that besides catches in the deep sea one very large haul of *Amphioxides* was made near shore in a region inhabited by *Asymmetron lucayanum*. Though the anatomical details of the two forms did not agree completely, Gibson holds that *A. pelagicus* is the larval form of *Asymmetron lucayanum*. (My last-mentioned paper, reporting the metamorphosis, was not yet known to the author.)

Table I

Dr. Beebe's material consisted of 87 specimens, of which 73 were available. When number of slits or length is not stated, the condition of the specimen did not permit an exact statement.

Haul No.	Depth *meters*	Date	Remarks	Number
253	1829	4/7/29	7 mm., 20 slits	1
357	1463	9/8/29	Macerated	1
363	1829	10/8/29	11 mm., 22 slits	1
386	1280	17/8/29	14 mm., macerated	1
400	1829	31/8/29	All ca. 8 mm., 18–22 slits	41 (27)
401	2011	31/8/29	Macerated	1
404	1097	2/9/29	Ca. 10–12 mm., badly preserved	11
406	1463	2/9/29		
407	1646	2/9/29		
498	7	23/9/29	Macerated	1
519	0–183	30/9/29	Typical	1
524	0–1097	30/9/29	Typical	1
735	1829	27/6/30	23 slits	1
761	1646	2/7/30	Macerated	1
791	914	9/7/30	Macerated	1
818	1097	29/8/30	15 mm., 26 slits	1
835	914	3/9/30	Macerated	1
875	1097	11/9/30	Macerated	1
907	0–183	18/9/30	Typical	3
909	0–549	18/9/30	21 slits	2
910	0–732	18/9/30	8–10 mm., 24 slits	7
912	0–914	18/9/30	22–26 slits	3
913	0–1463	18/9/30	14 mm., 24 slits	1
1063	549	8/7/31	20 slits	1
1193	914	17/8/31	Macerated	1
1301	92	15/9/31	21 slits	1
1344	0	3/11/31	20 slits	2

Though we know now that *Amphioxides* is a larval form, able to metamorphose at an uncommonly late stage, we do not know whether this is the ordinary type of development of certain forms like *Asymmetron*. After more than twenty years I was therefore much gratified to be able to return to this old topic during a stay at the Bermuda Biological Station. Dr. William Beebe was kind enough to let me have all the *Amphioxides* which he had caught near the Bermuda

Islands during his expeditions for the New York Zoölogical Society, and the Director of the Station, Dr. Wheeler, gave me the opportunity to compare this material with the living *Asymmetron* of this region. I should like to thank both gentlemen for all courtesies. The point in question was whether the *Amphioxides* forms near the Islands could be shown to coincide with the native *Asymmetron lucayanum.*

The *Amphioxides*-material was caught about five miles off the coast of Bermuda Islands with deep sea nets,[1] the contents of which were supposed to have been caught mainly at the depths stated in Table I. But whereas no closing net was used, these data are not beyond criticism. They agree, however, with the data of former work which show the occurrence of *Amphioxides* from the surface down to rather considerable depths. In the majority of instances only one or a few individuals are reported. But one haul netted 41 specimens at once,

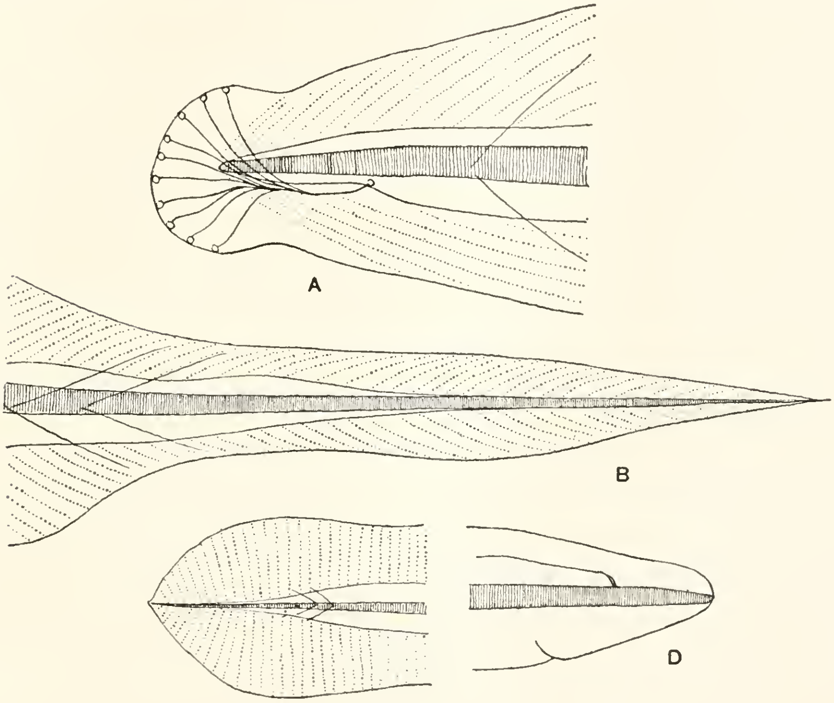

FIG. 1. *A.* Outline sketch of head of *Asymmetron lucayanum* from Bermuda. *B.* Tail of same. *C.* Outline sketch of head of *Amphioxides pelagicus* from Bermuda. *D.* Tail of same.

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[1] For details see Beebe, W., 1931. Bermuda Oceanographic Expeditions. *Zoölogica,* **13.**

an occurrence which is paralleled in the material of the British Expedition. The size of the specimens varies between 8 and 16 mm. All of them belong to the same species, namely, *A. pelagicus*. The gill-slits do not increase regularly with size. It seems that at a stage of ca. 8 mm. almost the maximum number of gill-slits is already formed and only a few may be added afterwards. At this stage 18–22 slits are found and the largest individual possessed only 26. This again agrees with former findings. The decisive systematic character for comparison with other forms is the number of segments. In all suitable specimens I counted 50–51 pre-anal and 12 post-anal segments. This count does not agree with the typical *A. pelagicus*, which has 53 pre-anal and 14 post-anal segments (see later).

The typical *Asymmetron lucayanum* of the Caribbean has, according to literature, $53 + 13 = 66$ segments. In the Bermuda form I have counted 67 segments which would agree with the Pacific *A. pelagicus* but not with the Caribbean. After the segmentation the most characteristic feature of *A. lucayanum* is the form of the snout and of the tail-fin. Both are absolutely different from the *Amphioxides* forms, as the figure shows. The alternative conclusions then are: either the Bermuda *Amphioxides* do not belong to *Asymmetron* or they are able to change their shape and segmentation completely during metamorphosis. The latter seems rather improbable.

There also exists in the Bermudas a species of *Branchiostoma*, *B. bermudae* Hubbs. But its myotome number is only 54–57, which excludes any relation to the *Amphioxides*. The relation between *Amphioxides* and the *Amphioxus*-forms of the nearest littoral region is therefore unsettled in this case also.

The actual situation then is:

1. *Amphioxides* forms are found all over the world (see map in Goldschmidt, 1909).
2. At least three clearly distinct types can be distinguished, namely *pelagicus* Gill, *valdiviae* Goldschmidt, *stenurus* Goldschmidt.[2] Probably *pelagicus* consists of more types (*see* the Pacific form with 67 segments, the Bermuda form with 62–63, and the North Sea form certainly belonging to *B. lanceolatum* Pallas).
3. *Amphioxides* forms are found in the littoral zone (one case of Gibson) and on the high seas from the surface to the deep sea.
4. *Amphioxides* forms grow and differentiate far beyond the normal stages of metamorphosis found in the littoral zone.
5. They are able to metamorphose in far advanced stages of growth and differentiation, as shown for *A. valdiviae*.

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[2] Not *sternurus*, as Gibson and Hubbs write,—stenos = narrow.

6. Occasionally the development of gonads begins before metamorphosis and proceeds rather far.

7. In those cases which are known thus far, only right gonads were formed, which is typical for the genera *Epigonichthys* and *Asymmetron*.

8. Thus far no *Amphioxides* could be assigned with absolute safety to a neighboring littoral species with the exception of the specimen from the North Sea belonging to *Branchiostoma lanceolatum*.

After taking all these facts together I come to the following conclusions: It seems that all *Amphioxi* have (always or under certain conditions?) two types of larvæ: one metamorphosing *in loco* and continuing the community in a given littoral region; another leaving the littoral for a more or less prolonged pelagic life with a much later metamorphosis, thus enabling the spread of the species. Biologists living in an *Amphioxus* region ought to be able to test this suggestion.

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DIURNAL CHANGES IN ACTIVITIES AND GEOTROPISM IN THYONE BRIAREUS

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I

In *Thyone briareus* waves of constriction completely encircling the body and involving both bivium and trivium travel along the longitudinal axis of the body. These waves usually serve as a means of locomotion and aid in extruding the tentacles. However, at times waves course the whole length of the body without producing any progression of the animal. Whether waves pass toward the oral or aboral pole depends upon the presence of certain forces in the environment of the animal and upon the incidence of periods of activity in *Thyone*. The following experiments, investigating the control of the locomotor waves, were made at the Marine Biological Laboratory, Woods Hole, during January, 1928, and since then have been repeated at various times in the Laboratory of General Physiology, Cambridge.

II

At 16.0° C. (7:00 P.M., in winter) a new wave of constriction originated at one pole about every four minutes (4.3 minutes average) and progressed with a uniform speed (0.5 mm. per second at 16.0°; 0.9 mm. per second at 25.5° C.) toward the opposite end. The rate of progression was practically the same in either direction (oral to anal, 0.87 mm. per second average: anal to oral, 0.89 mm. per second at 25.5° C.). During the morning and early afternoon few locomotor waves were seen, also fewer animals extended their tentacles. Beginning at about 4:00 P.M. (winter months, Woods Hole) more waves were seen and more animals had their tentacles extended. The same periodicity of activity was noticed even when the animals were kept in a dark room and viewed by red light. This state of increased activity continued until about 1:00 or 2·00 A.M.; thereafter the animals exhibited fewer locomotor waves and the tentacles remained retracted.

When the tentacles were not extended the waves of constriction usually originated at the oral end and coursed toward the anal region; when the tentacles were extended the waves started at the anal end and traveled toward the oral pole. It is as if the waves originated at that pole exhibiting the lesser activity. When the oral end is quiet the anal end is, of course, relatively more active since at that end the respiratory movements go on all of the time.

While it is usual to find at any moment only one wave traversing the animal, two waves have been seen in some instances going in the same direction; occasionally two waves were seen going in opposite directions. Sometimes waves were seen to originate simultaneously at both ends of the animal and approach each other. At the meeting point, one of the waves disappeared; the other one continued its course to the anal end. Such phenomena frequently occur in animals possessing a nerve net. (Mayer, 1906, 1908; Crozier, 1915; Child, 1917. In the intestine of mammals, Alvarez and Mahoney, 1924.) Apparently in *Thyone* there are at each end of the animal regions initiating the onset of locomotor waves.

While the animal is in a horizontal position, waves can be started experimentally at almost any place along the longitudinal axis of the body by producing a tension in the selected region. All experiments were made in the evening. (1) A small region of the bivium of animals which had attached themselves to a substratum was loosened and a glass plate of 1 cm. thickness and 1 cm. width was slipped beneath the animal. (2) A 45° glass prism was placed under the animal so that it fastened itself over the angular edge of the prism. (3) A hæmostat was attached to the tube feet of the trivium and the animal was freely suspended in sea water in a horizontal position. (4) *Thyone* was placed in a beaker small enough to cause the animal to take up a U-shaped form. In all these experiments the waves of contraction appeared at the site of stretch or tension. This was also found to be true for *Thyone* which had recently eviscerated.

A local tension brings about a contraction which then travels toward one or the other end of the animal. Experiments by Henderson (1928) on peristalsis of the guinea-pig and rabbit intestine show that nerve cells arranged in short reflex arcs are concerned in the peristaltic mechanism of this organ. Slight increments in pressure were found sufficient to elicit a peristaltic wave. Cannon (1911, p. 187) concluded from his investigations on the same question that the muscle fibers which are stretched tend to contract. The basic rhythm of contraction in the intestine seems to have a myogenic origin (Alvarez and Mahoney, 1922).

Work by Tao (1927) suggests that the contraction of smooth muscle in holothurians is controlled myogenically. The appearance of locomotor waves in *Thyone* is similar to the beginning of peristaltic waves in the stomach, where the pacemaker controlling the frequency of appearance of waves seems to be located in the region on the lesser curvature next to the cardia (Alvarez, 1928, p. 106). In *Thyone* it is as if a pacemaker mechanism is controlling the formation of a new wave every four minutes (at 16° C.) at either the aboral or oral end. However, as was shown above, the waves can be initiated experimentally by the production of an appropriate tension in the body wall. It is still unknown whether the locomotor wave originates at one end of the animal and first makes its appearance as a constriction at the locus where the tension was applied, or whether the wave of contraction originates at the locus of tension. Alvarez (1928, p. 108) has recorded many instances where a wave is seen to originate at the cardiac end of the stomach, disappear from view entirely for a short distance, and then reappear at a region caudad to the origin. A few of my observations on *Thyone* agree with these findings of Alvarez. The animal was placed in a beaker so as to take up a U-shaped form; the waves originated at the locus of bending, travelling sometimes to the oral end, sometimes to the aboral end. When the tentacles were withdrawn the waves travelled aborally, when the tentacles were extended the waves travelled orally, starting in all cases from the locus of bending. One might think of the wave of contraction as originating at the pole exhibiting the lesser activity but not becoming visible until the region of tension was encountered. Very often waves of contraction starting at one end of the body suddenly faded out further along the axis of the animal. Whether the initiation of waves of constriction at either pole of the animal (when it is in a horizontal position) is controlled by tensions developed in these regions or whether an oral or aboral pacemaker sets off the wave of contraction is still an open question.

III

During the morning and early afternoon the origin of progression waves in *Thyone* is usually fixed at one of its poles (oral), regardless of which end pointed down. The tentacles were usually retracted during this part of the day. Such persistence of polarity continued until about 7:00 to 8:00 o'clock in the evening. When *Thyone* was then placed in a vertical position the waves were found to begin at the bottom, regardless of which end of the animal pointed down, and coursed upward and moved the animal in that direction. Under these experimental conditions *Thyone* can be thought to exhibit negative geotropism. After 1:00 A.M. the polarity remained fixed, usually at the oral pole. In these experiments glass plates to which the animals had attached themselves while in a horizontal position were rotated so as to bring the body axis into a vertical position. In alternate tests aboral and oral poles were placed at the bottom. In other experiments this procedure was repeated with V-shaped glass troughs; also with glass tubes. Even when the animal was suspended from either end by a small hæmostat attached to several tube feet, the waves continued their upward movement whether the oral or aboral pole was placed at the bottom. No mention of this curious diurnal alteration in the locus of origin of the waves was made by Pearse (1908) in his observations on *Thyone*.

Negative geotropism was only obtained regularly during the mid-winter months. The reversal of polarity of locomotor waves when the animals were rotated in the gravitational field was only occasionally obtained during the months of May, June, July, and August at Cambridge; during this time of year *Thyone* did not show a regular diurnal periodicity of activity.

The reversal of direction of the locomotor wave on the body of the animal according to the orientation with respect to gravity seems to give an instance of geotropic stimulation in which the structural polarity of the organism is of secondary consequence. Emphasis is placed upon the directional control of the wave movements, rather than upon locomotor progression of the animal, because the waves may be obvious without actual creeping.

A reversal of upward, geotropically-oriented creeping of tree snails such as *Liguus, Pleurodonte,* and others, has been brought about by Crozier and Navez (1930). The upward orientation was shown to be governed by the sensible equality of tensions produced through the pull of the body upon its supporting elements. Downward orientation on a vertical surface was simply obtained by properly adjusting the pull upon the shell (by a string attached to the shell or by a load of 5 grams attached to the shell). Under water *Liguus* also orients upward. Reversal of orientation under water on a vertical surface can also be obtained by attaching a cork float of appropriate size to the apex of the shell. In both these cases of reversal of orientation and in additional experiments on geotropic excitation in *Helix* (Hoagland and Crozier, 1931–32) it is difficult to conceive a mechanism dependent primarily upon a statocyst. These facts are entirely consistent, however, with the notion of proprioceptive stimulation through impressed tensions as the controlling feature in the gravitationally excited orientation (*cf.* Crozier, 1929).

Evidence presented above points clearly toward a deficiency of statocyst control of geotropism in Thyone, since no specific polarity is encountered in upward creeping. In fact, in an aquarium, equal numbers of the animals will be found with either pole uppermost. This was also observed by Pearse (1908). The absence of statocyst function is further suggested by experiments with eviscerated animals, which still can be made to exhibit negative geotropism during the late evening hours in the winter. In such animals the tentacles, the stone canal with its nerve ring, and the gut are thrown out in their entirety; the possibility of attributing the origin of these waves to tension on the mesentery of the viscera, as has been shown to be the case in the righting movements of starfish (Wolf, 1925) and as suggested by Parker (1922) for the orientation of the sea urchin *Centrechinus*, seems to be eliminated. The cloaca and the attached trachial trees have also been removed from eviscerated animals and they still always oriented upward, during the evening hours of winter months.

When the holothurian *Caudina chilensis* is imbedded in sand and is rotated (Yamanouchi, 1929), it responds to centrifugal force in such a manner that the oral end bends away from the center of rotation and the tail turns toward the center of rotation. Even when the anterior part of the body including the nerve ring is amputated the tail reacts to centrifugal force in the same manner as the intact *Caudina*. Statocysts are apparently not involved in these reactions. The responses seem to be brought about by differential tensions in the body wall produced by the rotations. The evidence from experiments on *Synaptula* (Olmstead, 1917) and *Cucumaria cucumis* (Loeb, 1891) suggests that a statocyst mechanism orients the animal so that its oral end is always upward while ascending a vertical plane by aid of its tentacles. Clark (1898, 1899) has described structures in the oral stone canal of *Synaptula* to which he has assigned the function of statocysts. No mention was made in these studies of any diurnal fluctuations in the animal's response to gravity, so we assume that it remains negatively geotropic throughout the entire day.

The sensitivity of the body wall of *Thyone* is so altered diurnally that the structural polarity of the body is of minor consequence when the animal is oriented by geotropic excitation. The deforming pressure of the pull of the body wall when the animal is on a vertical plane elicits a locomotor wave at its lower end. The direction of the waves is primarily determined by the force of gravity only during the evening, and then especially during the winter months; only at this period of the day and year have waves been found to originate at either pole and course upward.

SUMMARY

Thyone briareus observed during the winter months exhibited certain definite diurnal periods of activity. (1) Between about 4:00 P.M. and 1:00 to 2:00 A.M. the frequency of "locomotor" waves increased in number and the feeding movements of the tentacles were more numerous than at other periods of the day, even when observed in the dark under red illumination. (2) When during the late evening hours *Thyone* was rotated in a vertical plane, locomotor waves coursed negative to gravity regardless of which end of the animal was oriented downward. Since it was found that waves of contraction could be elicited experimentally at almost any region by producing tensions in the body wall when *Thyone* was in a horizontal position, it is supposed that the sensitivity of the body wall is so altered during the late evening hours that tensions produced in the body wall when the animal was placed in a vertical position are sufficient to bring about the formation of locomotor waves at the lower end. Absence of statocyst function is suggested by these experiments, and by additional experiments with eviscerated animals which still can be made to exhibit negative geotropism.

During spring and summer months the activity of *Thyone* was not so definitely marked off into diurnal periods, neither was the negative geotropic response regularly obtained.

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MONOCENTRIC MITOSIS WITH SEGREGATION OF CHROMOSOMES IN SCIARA AND ITS BEARING ON THE MECHANISM OF MITOSIS[1]

I. The Normal Monocentric Mitosis. II. Experimental Modification of the Monocentric Mitosis

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I. The Normal Monocentric Mitosis

It has become a generally accepted principle in cytology that a prime essential for complete mitosis is the presence of a bipolar or multipolar spindle (cf., *e.g.*, Wilson, 1925, p. 168; Gray, 1931, p. 172) and that in a unipolar field, although the chromosomes may divide, they cannot segregate into two groups. In flies of the genus *Sciara*, however, the first spermatocyte regularly undergoes as a normal process a monocentric mitosis which effects a regular and precise segregation of chromosomes (Metz, 1926*a*; Metz, Moses, and Hoppe, 1926). Since this process deviates widely from that observed in ordinary mitosis it has invited careful analysis, and has led to a series of studies involving several species.

Occurring as it does in the reduction division, the mitosis in question segregates homologous chromosomes. Consequently genetic as well as cytological methods have been used to trace the behavior of individual chromosomes (Metz, 1926*b*, 1927, 1928, 1929), and to determine the principle underlying the segregation process (see below). The present brief account is concerned primarily with evidence bearing on the mechanism of chromosome movement.

In the prevailing theories of mitosis[2] there has been a strong tendency to attribute the movement of the chromosomes to the poles in anaphase to forces of attraction or repulsion, or both, emanating from outside the chromosomes, and to consider the chromosomes themselves as playing a relatively passive rôle, or at most as exerting a mutual repulsion.

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[1] The second part of this investigation has been aided by a grant from the National Research Council, Committee for Research on Problems of Sex. The writer is indebted to Mrs. Philip B. Armstrong (M. Louise Schmuck) for assistance in that part of the investigation.

[2] See, *e.g.*, Wilson, 1932; Sharp, 1926; Bělař, 1929*a*, *b*, *c*; Gray, 1931.

So far as I am aware, no significant indications have been observed in ordinary mitoses that autonomous activity on the part of the chromosomes themselves plays an important part in their anaphase movements,[3] although various lines of evidence suggest that they may exhibit such activity at other stages.[4]

In the monocentric mitosis in *Sciara* the anaphase movement of certain chromosomes exhibits characteristics which strongly suggest that it is due in large part to the activity of the chromosomes themselves. These characteristics will be considered briefly below.

As noted in the papers referred to above, the monocentric mitosis in *Sciara* also presents evidence bearing on the much-debated question of the rôle played by the so-called " spindle fibers " in mitosis. It indicates that, whatever their precise nature may be, they represent a functional reality and reflect an activity operating on the chromosomes in the direction of the pole. (The active agent here may be the " insertion region " of the chromosome, or may be external to the chromosome.)

In the present studies observations have been made on both sectioned material and smears. In all cases the testes were dissected out and freed from surrounding material before fixation. On account of their small size, this permitted almost direct contact of the cells with the fixing fluid even in the case of the sectioned material. In the smears the cells were, of course, brought immediately into direct contact with the fixative. Excellent fixation was secured in both ways. Various fixatives have been used, but most of the observations have been made on material fixed in Gilson's mercuric-nitric mixture and stained in Heidenhain's iron haematoxylin. Flemming's fluid has been used, but with unsatisfactory results due to the presence of large chondriosomes which obscured the mitotic figure in most cells (see Metz, Moses, and Hoppe, Fig. 34). At the stage in question these chondriosomes usually form a saucer-shaped or cup-shaped mass lying peripherally and often extending about halfway around the cell. They have received special study by Mr. W. L. Doyle (paper in press).

Unfortunately details of the achromatic figure are not as satisfactorily brought out after fixation with Gilson as by other methods, and for this reason certain of the finer aspects of the figure have not yet been analyzed. The features essential for present purposes, however, are revealed clearly enough in the sections studied. In the smears the achromatic figure is so exceedingly delicate in structure as to be almost invisible, due presumably to the delicacy of the fixation and the similarity of the refractive indices of the materials in the figure. The general nature of the structure, however, is clearly indicated by the configuration and behavior of the chromosomes.

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[3] Since this was written, papers by Bleier (1931) and by Wilson (1932) have come to hand, both emphasizing the possibility of such autonomous activity. The literature on the subject is reviewed by both authors.

[4] See, *e.g.*, McClung, 1927; Lewis, 1932; Lewis and Lewis, 1932.

All the material is from pedigreed cultures which have been kept in the laboratory, in some cases for many generations.

The principal characteristics of the monocentric mitosis in *Sciara coprophila*, Lint. may be summarized as follows: (For details see Metz, Moses, and Hoppe, 1926.)

1. The spermatogonial chromosome group as shown in Text Fig. 1 typically consists of ten members: three pairs of rod-like chromosomes, one pair of small V-shaped chromosomes, and one pair of much larger V-shaped chromosomes (the " limited " chromosomes). The " limited " chromosomes are limited to the germ-line, due to elimination from somatic nuclei in early cleavage.[5]

2. No synapsis occurs. In prophase of the first spermatocyte division (Text Fig. 1) the univalent chromosomes appear to be distributed at random, but about equidistant from one another, about the periphery of the nucleus when the nuclear membrane breaks down.

3. After the nuclear wall vanishes a half spindle appears and the chromosomes, apparently without changing their locations, all become oriented toward the single pole, each with a " spindle fiber " extending from the normal insertion point on the chromosome toward the pole. No centrioles have been identified.

4. The chromosomes do not become arranged in any one plane and nothing comparable to an equatorial plate or metaphase stage is found. On the contrary, they move from their prophase positions either directly toward, or directly away from, the pole (Text Fig. 2).

5. Certain chromosomes regularly move toward the pole in the customary fashion. These include both of the large " limited " chromosomes and one member of each of the other four pairs of chromosomes. Genetic evidence indicates that among the latter four pairs it is regularly the maternal member which passes to the pole. This evidence appears to be complete for at least three of the four pairs in one species.[6]

6. The remaining four chromosomes—homologs of four which go to the pole—regularly move " backward " directly away from the pole, following radial, and hence diverging, paths as far as possible from the common center. When the chromosomes reach the periphery of the cell their courses are deflected and made to converge, ultimately bringing

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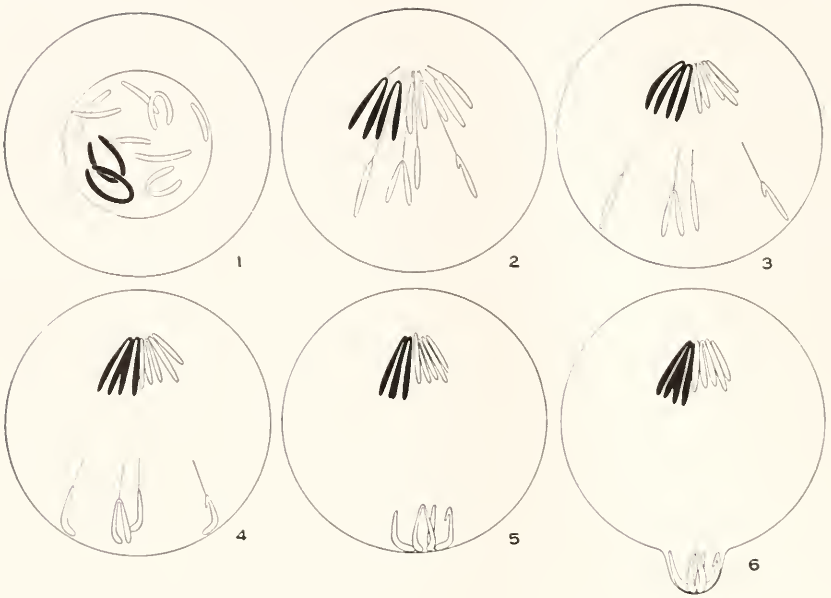
[5] See Metz, 1931; DuBois, 1932; Schmuck and Metz, 1932.

[6] Metz, 1927 and unpublished evidence of Dr. Helen B. Smith and the writer.



Text Figs. 1–6. Schematic representation of the movements of the chromosomes in the monocentric first spermatocyte division in *Sciara coprophila* Lint.

Diagram 1. Prophase just before dissolution of the nuclear membrane. The chromosomes are scattered, apparently at random, about the periphery of the nucleus.

Diagram 2. Beginning of migration of the chromosomes—six toward the pole and four directly away from it.

Diagram 3. Slightly later stage, after the six chromosomes have reached the pole and the other four have progressed to the periphery of the cell. The two median "retreating" chromosomes are presumed to be at high and low levels, respectively, and at the periphery.

Diagrams 4–6. Successively later stages showing the migration of the chromosomes along the periphery of the cell away from the pole, and the beginning of the extrusion process at the point farthest from the pole.

Actually the paths of the retreating chromosomes are only rarely equidistant from one another as shown in these diagrams (see text), and the chromosomes which pass to the pole do not ordinarily retain their polar orientation later than the stage shown in Diagram 3.

The two "limited" chromosomes are represented in black. These regularly pass to the pole and are retained. Among the other chromosomes precise segregation of homologs is effected. The hook at the proximal end of the retreating chromosome at the right, due to sub-terminal fiber attachment, is presumably characteristic of its homolog also, but is not evident in the latter, probably because of the lack of stretching. As will be seen from the camera drawings (Plate I), the retreating chromosomes are comparatively more elongate than the diagrams indicate. (See also Figs. 14–18 in Metz, Moses, and Hoppe, loc. cit.)

them together at a peripheral point opposite the single pole. Subsequently they are extruded in a finger-like process, where they degenerate (Text Figs. 3–6).

7. During their "backward" movement the four chromosomes retain their reversed orientation, moving with the spindle fiber insertion point hindmost, and the free end, or ends (in the case of the V-shaped member) foremost.

8. The posterior portion in these chromosomes is commonly taut and slender and often almost detached from the remainder, giving convincing evidence of a force, represented by the "spindle fiber," operating in the direction of the pole and in opposition to the movement of the chromosome (see Figs. 7 and 8).

9. In contrast, the apical ends of these chromosomes are thick, usually curved or twisted, and lie free, with no indication of any traction operating on them (see photographs, Plate I). It is clear that they are not being "pulled" in the direction of movement.

It is thus possible to distinguish the operation of two forces on the four retreating chromosomes. One is a retarding force, represented by the so-called "spindle-fibers" operating in the direction of the pole and only on one region of the chromosome, the insertion point. The other, and superior, activity operates in the opposite direction, and involves the chromosome as a whole, serving to cause the movement described above. It is the latter activity which seems to be performed mainly by the chromosome itself rather than by an outside agent.

As noted previously (Metz, 1926*a*), the chromosomes, during the movement in question, give the appearance of being carried by currents in the protoplasm. But careful study of a large amount of material prepared in various ways has convinced me that no true currents are involved and that the only flowing motion concerned is that of the material immediately around the individual chromosome. The details of the process will be presented in another paper. They may be summarized, however, by saying that each of the retreating chromosomes moves off independently, away from the pole, either in a small island of material (gel?) from the original half-spindle, or at the apex of a pseudopod-like outgrowth of this material.

Some idea of the independent movement of these chromosomes, and of the general aspect of the figures, may be gained from the drawings shown in Figs. 7, 8, and 10 and from the photographs in Figs. 15–17. For descriptions of these see explanation of figures.

That the movement is due to an activity of the chromosome itself is suggested mainly by two features. The first of these is the independent action of the individual chromosomes, each of which not only follows its own course, but follows a course apparently determined only by the original position of the chromosome in relation to the pole. This original position appears to be determined at random. If two or more of the four chromosomes happen to be adjacent at the beginning they move away together and follow essentially the same path. If they are well separated at first their paths diverge widely (see, *e.g.*, Fig. 17). Furthermore, the path of any individual away from the pole may follow any radial line within the theoretical cone delimited by imaginary extension of the half spindle. In other words, it seems impossible to explain the phenomenon on the basis of predetermined currents or lines of flow in which the chromosomes are caught and transported.

The second feature concerns the position of the chromosome in the island or process of " spindle " substance in which it moves. This position is not easily determined with accuracy, for only occasionally is the outline of the mass revealed. However, numerous large chondriosomes and other stainable constituents present in the cytoplasm sometimes delimit the clear mass distinctly. Careful study of such cases indicates that typically the position of the chromosome is apical. Since, as already noted, there is clear indication that the movement of the chromosome is retarded by an activity represented by the " spindle fiber," it seems improbable that the chromosome would remain at the apex of the moving mass unless it were itself responsible for the movement. In other words, were the chromosome simply being transported by an outward flow of the material from the half spindle, the retarding " spindle fiber " force would presumably cause it to move less rapidly than the surrounding material and hence not remain at the apex of the outflowing mass.

As a result of the above considerations and others of a more detailed nature, the hypothesis is advanced that the retreating chromosomes move because of their own activity. It may further be postulated that this activity operates by bringing about a progressive alteration in the physical state (*i.e.*, by solation or gelation) of the protoplasm adjacent to the chromosome.[7] Presumably the simplest form of such activity would be one producing a change in front of or behind the chromosome—the former serving to pull the chromosome along, so to speak, and the latter acting more as a pushing agent. Without attempting a detailed discussion of such possibilities, it may be noted in this connection that the chromosomes do not give the appearance which would be expected if the activity were operating solely on the anterior (distal) ends. This is particularly true in the case of the V-shaped member. Often the two arms of this V are deflected near the ends in opposite directions, yet they do not continue to move in opposite directions and pull the chromosome out into a straight line. Rather, the chromosome as a whole continues to move, even with the apices deflected. This would indicate that the activity is graded along the length of the chromosome—or, perhaps more probably, that it is an activity not only of the stainable portion of the chromosome proper, but also of the chromosome sheath, and hence that it involves a relatively large mass of material whose shape may be modified without seriously affecting the movement.

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[7] This hypothesis has been under consideration for more than two years, but has been withheld pending the completion of experiments which show that the chromosomes in question are alive and functional (see below). The possibility of bodies moving through the protoplasm by such means has been suggested to me by several physiologists, particularly Dr. Selig Hecht and Dr. S. O. Mast. To them and to Dr. Ralph S. Lillie I am greatly indebted for advice on numerous questions which have arisen in connection with the present study.

Any antero-posterior differentiation of the chromosome required by such hypothesis is readily conceivable, since the "insertion point" of the spindle fiber distinguishes the posterior end of the chromosome and may well influence the general activity of this region.

In the above considerations two possibilities have been omitted which may immediately be raised as objections or alternatives to the hypothesis presented. One is the possibility that the movements under consideration result from an electromagnetic activity and simply reflect a repulsion between the four retreating chromosomes and the pole. Perhaps such a possibility cannot be ruled out at present; but the many difficulties it encounters when applied to mitosis in general [8] make its validity seem very doubtful. For this reason it is being considered only as a last resort.

The other possibility is that the chromosome movements under consideration do not represent true mitotic activity at all. Since the four retreating chromosomes are ultimately cast off and degenerate, it may be suggested that they are already inert and degenerating during anaphase and hence that they are to be considered essentially as foreign bodies whose elimination has no connection with the process of mitosis. This possibility seems to be definitely ruled out by the experimental evidence considered below.

If the hypothesis of chromosome movement presented above is correct it should be applicable to ordinary bipolar mitosis. In such ordinary mitosis the two forces or activities here identified because of their antagonistic action would have to act in the same direction and supplement, instead of oppose, one another. This feature will be considered in a subsequent, more detailed, account.

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[8] See, *e.g.*, Wilson, 1925, p. 186; Gray, 1931, p. 167.

II. EXPERIMENTAL MODIFICATION OF THE MONOCENTRIC MITOSIS

As intimated above, when one studies the normal processes of monocentric mitosis in *Sciara* spermatocytes, he is at once confronted by the question as to whether the chromosomes which retreat from the pole are in a normal state of activity or are in a degenerating condition. The answer to this question bears not only upon the immediate problems involved in the present paper, but also upon other aspects of our studies—particularly those concerned with sex determination, the influence of sex on chromosome behavior, and the interrelation between cytoplasm and chromosomes. For this reason especial attention has been devoted to the subject and numerous experiments have been performed in an effort to modify the normal procedure in such a way as to give critical evidence. Such evidence has now been secured. It is considered below, together with other features bearing on the question at issue.

It is evident, as already indicated, that normally the chromosomes which are cast off in the polar-body-like process or "bud" eventually degenerate. This is true not only in the case of the monocentric first spermatocyte division, but also in that of the dicentric second spermatocyte division which, of course, immediately succeeds the first. The ultimate degeneration in both cases is readily understood, because the "bud" contains almost nothing but the chromosomes, and in the absence of cytoplasm these obviously could not long continue their normal development.

The immediate problem is to determine whether, in the monocentric division, this degeneration takes place after the "bud" is extruded or is already well under way during the division itself. Before considering the experimental evidence attention may be called to two features in the normal processes which strongly suggest that the former alternative is correct. The first of these is the fact that after the chromosomes are extruded in the "bud" they appear to undergo changes in form and structure similar to those exhibited by the chromosomes retained in the cell, although it is difficult to trace these activities in detail in the narrow compass of the "bud," and they apparently do not continue through more than the interkinesis period. They do seem to indicate, however, that the discarded chromosomes are not inert at the time they are discarded.

The second line of evidence comes from the second division. It seems clear that, although the chromosomes which are discarded at this division eventually degenerate, they are not degenerating during the mitotic process, for the mitotic figure here is bipolar, the chromosomes show essentially typical behavior, and those which are discarded are sister halves of those which remain in the functional cell (spermatid).

Similar evidence is, of course, provided by the well-known phenomena of polar body formation in eggs, which show that the ultimate fate of chromosomes is not necessarily an indication of their condition during the preceding mitosis.

The final, and apparently conclusive, evidence, however, comes from experiments which show that when retained in the cell, instead of discarded, the chromosomes under consideration (in the monocentric division) remain alive and active, and hence that they must be alive and able to function during the mitosis itself.

The evidence is briefly as follows: Under suitable conditions, such as exposure to low temperatures,[9] the formation of the polar-body-like protuberance may be inhibited at either the first or second divisions. In such cases the chromosomes which would otherwise be eliminated are retained in the cell and are thereby enabled to continue functioning and to give evidence of their condition.

In the monocentric mitosis it is apparently possible to stop the " backward " movement of the retreating chromosomes at any stage of what would correspond to anaphase in typical mitosis. The subsequent positions of these chromosomes, and to some extent their behavior, appears to be determined by the time at which their migration is stopped. This is inferred from the fact that in the preparations examined the chromosomes are in the various positions corresponding to those through which they normally pass during the monocentric mitosis.

The details of these features will be left for a more complete account. Present interest centers in the fact that the retreat of the chromosomes has been stopped at various stages and that preparations have then been made at various subsequent time intervals. In consequence it is possible to reconstruct the sequence of stages showing the behavior of these retained chromosomes during the ordinary transformations of the second spermatocyte.

Development of the second spermatocyte in this treated material apparently progresses in the normal fashion. The chromosomes of the regular nucleus go through the usual interkinesis, prophase, metaphase, and anaphase stages. Coincidently the four artificially retained chromosomes go through a corresponding series of stages up to late prophase or metaphase. They become diffuse or reticular like the others during the telophase and interkinesis (see Fig. 12); they divide, and they condense into split prophase chromosomes during the prophase of the second spermatocyte. They appear to undergo these transformations regardless of their position in the cell or the stage at which they were interrupted in the monocentric division. They also appear to act independently of one another, in the sense that they exhibit essentially the same characteristics when widely separated from one another as when close together (see Figs. 13 and 14). When separated each is surrounded by a relatively transparent layer of protoplasm, which I interpret as an integral, although perhaps transitory, part of the chromosome, representing the chromosome "sheath" described by numerous observers in ordinary mitoses.[10] When close together, the chromosomes give the appearance of lying in a small nucleus; but I have been unable to detect a nuclear membrane and am inclined to attribute the appearance to the coalescence or approximation of the transparent layers or sheaths around the chromosomes.

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[9] The precise nature of the treatment required has not yet been determined. The conditions described here were first found in material purposely subjected to cold. Similar conditions have been observed in a few specimens taken from laboratory stocks which had probably been exposed to low temperature through cooling of the room.

In most cases these artificially retained chromosomes do not exhibit an increase in size corresponding to that of the chromosomes in the normal nucleus. They vary considerably in this respect and the evidence as a whole suggests that the growth is dependent on the position of the chromosome with respect to the normal nucleus—being greatest when there is a close proximity (see Fig. 14). This in turn suggests that the nature of the protoplasm immediately surrounding the chromosome is an essential factor in respect to growth, and that only the environment provided by the nucleus itself is suitable for promoting normal growth.

In no case has it thus far been possible to inhibit the monocentric mitosis in such a way as to retain all the chromosomes in the nucleus and thus get a second spermatocyte nucleus containing the total diploid group; but it seems almost certain from the above evidence that if this could be done the growth and activity of the chromosomes which are ordinarily eliminated (paternal members) would be equivalent to that of their (maternal) homologs.

As would be expected, the correspondence between the transformations of the retained chromosomes and the others ceases at the metaphase of the second spermatocyte division. In the absence of any mitotic apparatus (asters or spindle) the daughter halves of the artificially retained chromosomes cannot separate, hence they remain together during the anaphase of the second division. Whether or not they would subsequently exhibit further activity during the development of the spermatid has not been determined.

From the evidence presented above it seems clear that in the monocentric mitosis under consideration the retreating chromosomes are alive and are capable of taking an active part in the mitotic movements as postulated in the first part of the paper. This does not, of course, mean that they are necessarily entirely unaffected by the monocentric mitotic phenomena under normal conditions, or that their activity is precisely like that of their homologs which go toward the pole. Indeed, it seems evident that a difference in activity does exist, that it is due to the sex of the parent from which the chromosomes were derived, and that it is in some way responsible for the opposite responses of the two sets of chromosomes.

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[10] A good example is described by Metz and Nonidez, 1924.

Summary

I

The present paper supplements previous studies on the monocentric first spermatocyte division in *Sciara* and considers the findings in relation to the general problem of the mechanism of mitosis. Contrary to generally accepted principles of mitosis, an accurate segregation of chromosomes is effected in a unipolar field during the mitosis in question. The segregation is highly selective in that paternal chromosomes react in opposite fashion from their maternal homologs and pass away from the pole instead of toward it. These chromosomes which " retreat " from the poles exhibit characteristics which are believed (1) to demonstrate the functional reality of the so-called " spindle fibers," which in this case retard the movement of the chromosomes, and (2) to suggest that the opposing force or activity responsible for the movement of the chromosomes is due to an activity of the chromosomes themselves. The hypothesis of " autonomous " movement on the part of the chromosomes is suggested particularly by two lines of evidence:

(1). Each retreating chromosome in its movement directly away from the pole acts independently of the others and may move along any radial line within the theoretical cone delimited by imaginary extension of the half spindle. Its path appears to be determined entirely by its original position with respect to the pole.

(2). Each retreating chromosome appears to move in a small mass of protoplasm derived from the original half spindle, and to maintain its position at the *apex* of this mass during the movement. On account of the opposing activity represented by the so-called " spindle fiber," it seems improbable that the chromosome could retain this apical position if it were not itself responsible for the movement.

The suggestion is made that the anaphase movement of chromosomes may be primarily due to such activity of the chromosomes themselves and that this activity serves to bring about movement by producing localized alterations in the viscosity of the adjacent protoplasm.

II

Experimental evidence is reviewed which shows that the retreating chromosomes are alive and therefore theoretically capable of function-

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ing as postulated during the movements in question. This evidence also bears on the interrelations between sex and chromosome behavior to be considered subsequently.

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Plate I

All figures are from spermatocytes of *Sciara coprophila* Lintner, fixed in Gilson's mercuric-nitric fluid and stained in Heidenhain's iron haematoxylin. Figures 7–14 are from camera lucida drawings made at table level, using 1.5 mm. objective and No. 12 ocular. They are reduced approximately three-fifths in reproduction. The drawings were made by Dr. Esther Carpenter, with the exception of that for Fig. 11 which was made by Miss Louise H. Buck. Figures 7–10 are from untreated specimens showing certain features of the normal monocentric mitosis; Fig. 11 from a specimen treated with cold; Figs. 12–14 second spermatocytes from specimens presumably exposed to cold in the laboratory during the first division. Figures 7–10 are from sections; 11–14 from smears.

Fig. 7. Primary spermatocyte in approximately the stage represented in Text Fig. 4. The group of six chromosomes has reached the pole and lost its earlier orientation. The four retreating chromosomes have begun to converge after reaching the periphery. Note the two chondriosomes separating the V-shaped chromosome at the left from the others and showing that this chromosome has moved through part of the chondriosome mass (only part of which is shown).

Fig. 8. Slightly later stage, corresponding approximately to that shown in Text Fig. 5. The chromosome in black at the right has apparently passed through the mass of chondriosomes along the path indicated. The chondriosomes in the center lie at about the level of the chromosome at the extreme left, which may have passed through the opening between the two clusters.

Fig. 9. Slightly earlier stage than the preceding. In this cell the four retreating chromosomes are not widely separated. They have come into contact with the saucer-shaped mass of chondriosomes preparatory to passing through it. The contact has evidently served to bend or fold their distal ends, giving a clumped appearance. This is shown clearly by the V-shaped chromosome at the left. Although not very evident in the figure, the transparent sheath around each chromosome is distinct in the preparation; it serves to keep the chromosome proper from touching the chondriosomes.

Fig. 10. Incomplete figure at approximately the same stage as the preceding. Three retreating chromosomes are shown—two rods and the V. The one at the left is at a higher level than the others and is completely cut off from the periphery by chondriosomes, only a few of which are shown. The sheath surrounding this chromosome is in contact with the chondriosomes. This chromosome would subsequently have passed between the chondriosomes to the periphery. Such cases seem to demonstrate that the chromosomes are not transported by currents and that each is at the apex of the outflowing mass of protoplasm in which it lies, if there is any such mass in addition to the transparent "sheath."

Fig. 11. Primary spermatocyte from smear made from material treated with cold for 48 hours and dissected and fixed in cold. Cell slightly flattened. The four retreating chromosomes are long and slender and widely divergent—the V-shaped one lying at the upper left of the figure. Whether or not their elongation is due to the cold is uncertain, but it is probable that their movements were greatly slowed down, if not stopped, by the treatment. The chromosome at the lower right lies partly among the chondriosomes and is curved partially around one of them as shown in the figure. The achromatic figure is not visible in this cell.

Figs. 12–14. Second spermatocytes in which the chromosomes which should have been discarded at the first division have been retained, presumably due to exposure to cold.

Fig. 12. Interkinesis stage showing the chromosomes of the regular nucleus above and the artificially retained ones in a cluster resembling a nucleus below. The latter chromosomes are exhibiting the same interkinetic changes as those in the regular nucleus.

Fig. 13. Later stage from same testis as the preceding, showing the regular chromosomes, above, in metaphase of second division, seen in side view (compare with figures in Metz, Moses, and Hoppe, 1926) and the artificially retained chromosomes near lower right. The latter have divided and condensed and are in essentially the same stage as the others. Each is surrounded by its transparent "sheath," which in this case is clearly revealed by the relatively dark cytoplasm surrounding them.

FIG. 14. Later stage (middle anaphase) of second division from same testis. As in preceding three cases (all from smears) the achromatic part of the mitotic figure is practically invisible (see above under "methods"). The position of the spindle is clearly indicated, however, by the orientation of the chromosomes passing to the poles (for explanation of this mitosis see Metz, Moses, and Hoppe, 1926). The four lowermost chromosomes, all split, are the artificially retained ones—marked *r*. They lie near, but not in, one end of the spindle. The one shown in solid black lies at a high level directly above this pole of the spindle. Note that in this cell the retained chromosomes appear to be fully as large as those from the regular nucleus. In the cells of this testis only one large "limited" chromosome is present—a condition frequently found. The daughter halves of this chromosome in the present figure lie at the left of the spindle, going to opposite poles. The daughter halves of the small V are at the right of the spindle.

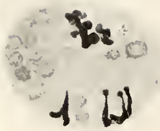
FIGS. 15–17. Photographs with explanatory drawings of three primary spermatocytes during the monocentric mitosis; from sections.

FIG. 15. A cell showing the retreating chromosomes shortly after they have reached the periphery and their paths have begun to converge. The chromosomes at the pole have lost their orientation and become irregularly clumped. *a*. Outline camera drawing; note that the V-shaped chromosome is at the right. *b*. Composite photograph taken at different levels to show relative horizontal positions of the retreating chromosomes. The two outer ones are at a high level and the two inner ones at a low level. *c* and *d*. Photographs at high focus and low focus, respectively, showing the features just mentioned.

FIG. 16. Similar illustrations of a cell at a slightly later stage. The V-shaped chromosome, near middle of the retreating group, and the rod at the right are at higher levels than the other two. *a*. Camera drawing (see Fig. 17, Metz, Moses, and Hoppe, 1926). *b*. Photograph at high focus showing the two upper chromosomes, with indications of the other two which are not entirely out of focus. *c*. Photograph at low focus, using higher magnification, showing the two lower chromosomes. As indicated by the figures, the paths of the retreating chromosomes are converging. The two lower members are moving upward and toward the right; the V is moving downward, and the rod at the right is moving downward and toward the left.

FIG. 17. Similar illustrations of an unusually small cell at an earlier stage than either of the two preceding. Three of the retreating chromosomes, two rods and the V, are readily seen at short distances from the other chromosomes, which are close together. *a*. Composite photograph taken at different levels. *b*. Outline camera drawing designed to supplement the composite photograph in showing the general composition of the figure, and the comparative horizontal positions of the chromosomes. Apparently the fourth retreating chromosome is the long one projecting from the compact group down toward the retreating V. This member is not completely separate from the group which has gone to the pole, although it lies below most of the members of this group. The identity of this chromosome is not certain, however, because the cell may be cut, and also the clustered chromosomes are too closely clumped to make individual identification possible. Note the wide divergence of the three conspicuous retreating chromosomes. Presumably none of them has quite reached the periphery. *c*. High focus showing the retreating hooked rod, at left. *d*. Lower focus showing the group near the pole and also indicating the vertical distance between the retreating rod just mentioned and the two other members shown in the next figure. *e*. Still lower level showing the retreating V and the rod seen at the right in *a* and *b*.

PLATE I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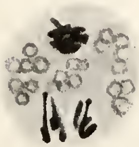
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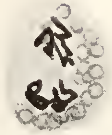
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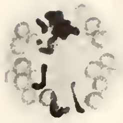
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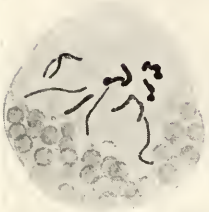
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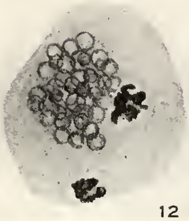
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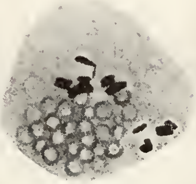
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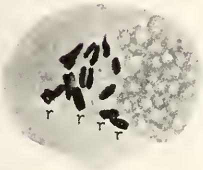

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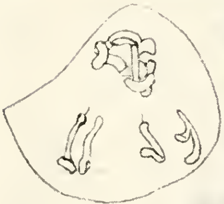
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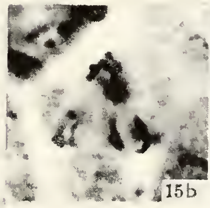
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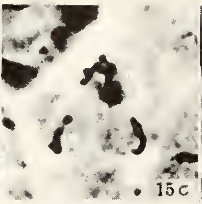
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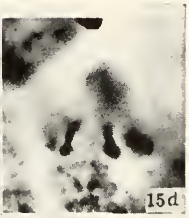
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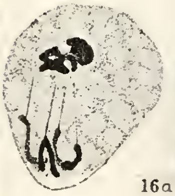
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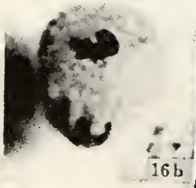
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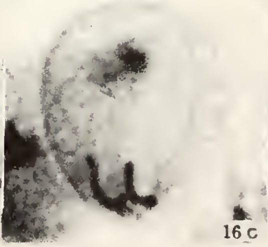
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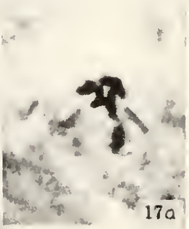
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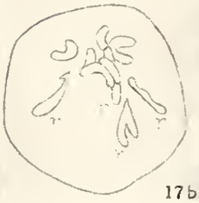
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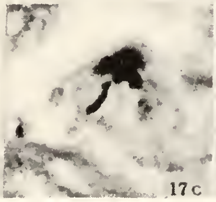
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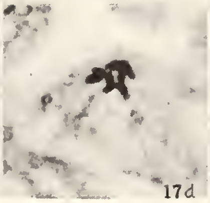
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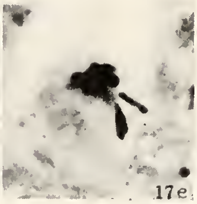
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CHANGES IN SUSCEPTIBILITY OF DROSOPHILA EGGS TO ALPHA PARTICLES

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In a previous report (Henshaw and Henshaw, 1933) we have shown that *Drosophila melanogaster* eggs vary markedly in susceptibility to 200 kv. X-rays at different stages during early development. A correlation of the changes in radiosensitivity with the changes in development showed that they become more sensitive during cleavage and blastulation, much more resistant at or near the beginning of gastrulation, and more sensitive again as gastrulation gets under way. Having found that susceptibility to one type of radiation varies in a certain way, it is of interest to determine whether it varies in the same way when other types of radiation are used.

Alpha particles, beta particles, and gamma rays are the three recognized forms of radiation emitted by radioactive substances. The alpha and beta rays are corpuscular in nature while the gamma radiation is like X-rays of very short wave length. All of these forms of radiation, including X-rays, are capable of causing ionization in matter subjected to their influence. According to the Rutherford-Bohr theory of the structure of the atom, the alpha particle is identical with the helium atom stripped of its two planetary electrons. It therefore has an electric charge of two positive and a mass about 7000 times that of the beta particle or electron (Failla, 1927). Since it has atomic dimensions and has a velocity of .15 to .20 times that of light, it represents an enormous concentration of kinetic energy. "In fact the alpha particle is the most potent agent known to science" (Lind, 1928).

In addition to the 200 kv. X-rays used previously, we have had at our disposal 40 kv. X-rays, gamma rays, and alpha particles. Tests determining susceptibility changes have been made with each of these. The responses to 40 kv. X-rays and gamma rays were essentially the same as to 200 kv. X-rays. With alpha particles, however, the effects were very different. Experiments with alpha particles will therefore be presented in detail, after which results for all will be compared.

ALPHA RADIATION

The source of alpha particles was polonium, which is one of the radioactive products of radium. Polonium was extracted from the walls of old radon tubes which had been used in cancer therapy. This was done with a dilute acid solution (.2 per cent HCl, approximately). The extract solution was flooded onto one surface of a copper disc 3.5 cm. in diameter and the polonium deposited on the metal surface. The amount retained by the disc was sufficient to kill *Drosophila* eggs after several minutes exposure. That alpha particles were responsible for the killing observed and not some other form of radiation was made certain by experiments which will be described later.

TREATMENT OF EGGS

A large collection of eggs was obtained from actively laying flies during a two-hour period.[1] This was divided into several portions, usually fifteen, each containing more than 100 eggs and often more than 200. With a soft brush the eggs were spread evenly in a single layer on small pieces of filter paper and were then ready for treatment.

The exposures were made in moist chambers, arranged by placing moist filter paper on the bottom of Petri dishes 15 cm. in diameter. The filter paper strips carrying the eggs were placed on these near the center. The copper disc was fastened in the center of the Petri dish cover so that the plated surface was directed toward the eggs and was about two centimeters above them when the dish was covered in the usual way. Eggs placed directly under the disc were killed by the radiation while those placed 5 cm. or more away were unaffected.

In order to determine the susceptibility of the material at different ages, tests were performed on different parts of the same collection of eggs at hourly intervals. The first began 15 minutes after the end of the collection period. Four samples were used for each test; the first three received 15, 30, and 45 minutes exposure, respectively; the fourth was kept as a control. Three exposures were used in order that the effect of different amounts of radiation could be considered. After treatment the samples and the control were put away in moist chambers at room temperature (22–25° C.). On the third day after irradiation, counts were made to determine the percentage hatched. This served as the criterion of effect.

RESULTS

The data obtained are shown in the accompanying table. Since age in development is the point in question, age is given relative to the time of fertilization rather than the time of collection. Since the eggs are fertilized individually as they pass through the vagina of the female fly and are deposited at any time during the collection period, their age is known to vary as much as two hours. It is necessary, therefore, to speak of an average age for the eggs in a sample. As pointed out in the

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[1] For culture of flies and the collection of eggs, see the earlier report—Henshaw and Henshaw.

TABLE I

Data on Changes in Susceptibility of Drosophila Eggs to Alpha Particles

	Exp. No	Age in Hours							
		1¼	2¼	3¼	4¼	5¼	6¼	7¼	8¼
		Percentage Eggs Hatching							
Controls	1	86	93	96	94	87	93	93	93
	2	91	92	88	89				
	3	90	94	96	91	95			
	4	97	93	91	96	92			
	5	93	99	93	89	98			
	6	98	97	99	99	98			
Average.		92	93	94	93	94	93	93	93
15 min. exposure	1	90	42	11	37	74	74	82	
	2	84	35	5	30				
	3	88	42	33	41	50			
	4	88	23	13	34	52			
	5	89	72	28	66	89			
	6	83	21	4	19	49			
Average.		87	39	16	38	52	74	82	
30 min. exposure	1	81	22	5	15	32	44	83	81
	2	81	22	4	10				
	3	85	18	20	22	19			
	4	71	10	5	8	28			
	5	46	35	29	22	84			
	6	71	10	6	6	17			
Average.		76	19	11	14	36	44	83	81
45 min. exposure	1	86	1	3	11	21	35	60	61
	2	71	9	1	4				
	3	68	11	18	19	14			
	4	56	12	11	5	9			
	5	49	23	26	18	79			
	6	45	8	10	6	29			
Average.		63	11	11	11	30	35	60	61

earlier report, this is approximately the middle of the laying period. Thus if age is reckoned from the average time of fertilization, the eggs are one hour old at the end of collection.

As seen from Table I, a control sample was kept for every age group irradiated. Among these the fertility varied from 87 to 98 per cent. The average, therefore, was 92 per cent; this was taken as 100 per cent and other values were adjusted accordingly. The average values were thus treated and plotted in Fig. 1 (solid line curves).

The abscissa (Fig. 1) shows age in hours and stage in development, while the ordinate shows the percentage of eggs hatching. The curves, therefore, show the percentage of eggs hatching at different ages after receiving 15, 30, and 45 minutes exposure to alpha particles. It will be seen that the eggs become increasingly sensitive during the first two or three hours after fertilization, after which time they become more resistant again. The point of maximum sensitivity (minimum resistance), therefore, is reached between 2 and 4 hours after fertilization.

The dash line curves show data obtained previously for 200 kv. X-rays. The shape and position of such curves vary depending upon the dosage of radiation administered. Accordingly, those for 2.5 (*A*) and 5 (*B*) minutes exposure (234 rœntgens per minute) were selected to show the most prominent changes. In the previous experiment a series of different dosages were used, but without exception there was a distinct increase in resistance between 2 and 4 hours after fertilization. The subsequent fall in resistance shown by curve *B* appeared only when larger dosages were given. By comparing directly the curves for the two types of radiation it is seen that, in general, they vary just opposite. While the eggs become more resistant to 200 kv. X-rays, they become more susceptible to alpha particles; the point of greatest resistance to 200 kv. X-rays and the point of greatest susceptibility to alpha particles being reached at about the same time.

Less complete data for 40 kv. X-rays (soft X-rays) and gamma rays are shown in Fig. 2. Only one dosage was used in each case. The curves for these radiations are similar to the one for 2.5 minutes exposure to 200 kv. X-rays. Presumably, therefore, if dosages had been varied for these radiations as for 200 kv. X-rays, similar variations due to dosage would have been observed. Whether this is actually the case can be determined only by further experiment, but the evidence is sufficient to show clearly that the organisms become more resistant to 40 kv. X-rays, 200 kv. X-rays, and gamma rays when they become more susceptible to alpha particles.

The problem, therefore, is to account for the difference in response to alpha particles. Since the response varies for different radiations

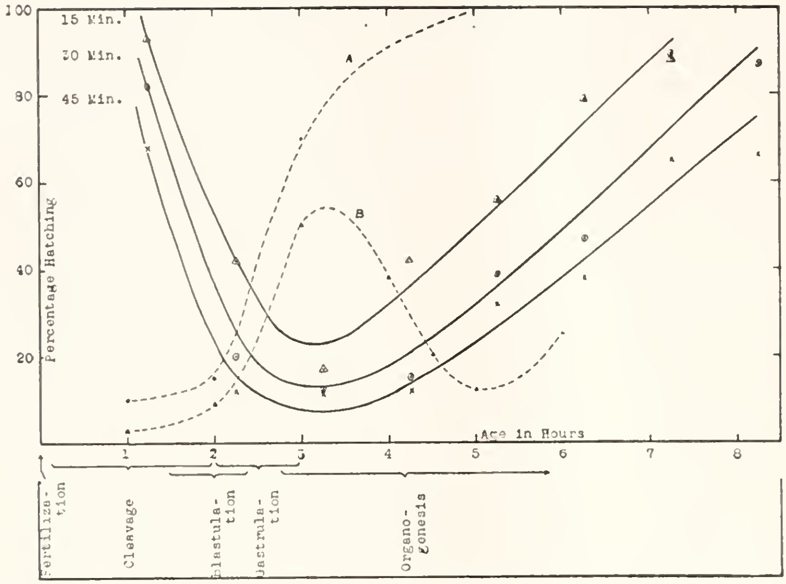

FIG. 1

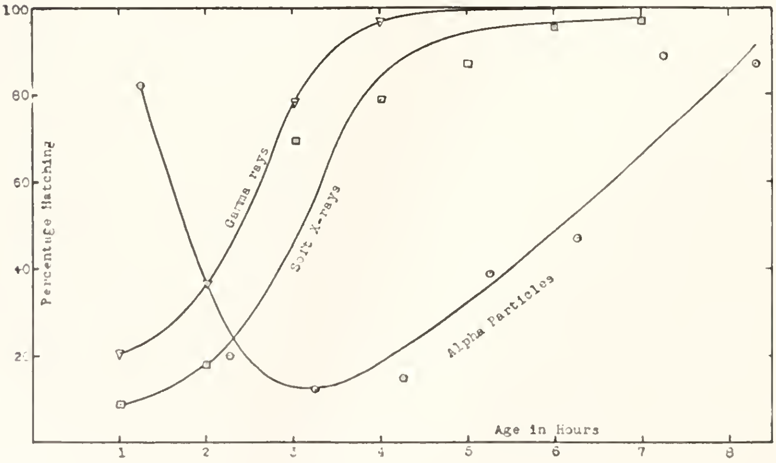

FIG. 2

FIG. 1. Curves showing changes in susceptibility to alpha particles (solid line curves) and 200 kv. X-rays (dash line curves). The index at the base of the figure shows the stage in development at different ages.

FIG. 2. Curves showing the changes in susceptibility to 40 kv. X-rays (soft X-rays), gamma rays, and alpha particles.

and different ages, it is evident that both the character of the radiation and the processes of development are involved. These will be discussed in turn.

PROPERTIES OF THE RADIATIONS

One of the essential differences between the radiations used is the power of penetration into matter. "As a rough working rule, it may be taken that beta particles are about 100 times as penetrating as alpha particles and that gamma rays are from 10 to 100 times as penetrating as the beta rays. . . . A thickness of .006 cm. of aluminum or mica or a sheet of ordinary writing paper is sufficient to absorb completely all the alpha particles" (Rutherford, Chadwick, and Ellis, 1930). Since, therefore, the large supply of kinetic energy possessed by the alpha particles is expended in so short a distance, much greater destruction is caused per unit length of their trajectory.

Alpha particles of polonium are completely stopped by 4 cm. of air, while beta particles require 5 mm. of aluminum or 1 mm. of lead to stop all (Lind, 1928). Gamma rays, on the other hand, will pass through 15 cm. of lead and the softer X-rays will pass through 1 mm. or more of aluminum. It is certain, therefore, that gamma rays and X-rays and some beta particles are capable of penetrating to all parts of the *Drosophila* egg. Unfortunately, microscopic preparations of eggs which have been exposed to alpha particles do not show delimited changes which mark the extent of penetration.

Alpha particles from polonium penetrate water a distance of 32 μ (Michl, 1914). Presumably, they penetrate about the same distance into protoplasm, since it has approximately the same density. Feichtinger (1931) reports that alpha particles of polonium produce visible effects in root tips to a depth of 30 μ. Zirkle (1932) described the effects of this radiation on the spores of the fern, *Pteris longifolia,* which have a diameter of about 38μ. The nucleus in this form ordinarily lies to one side of the center. When irradiated with the nucleus directed away from the source of radiation the effect was very different from that obtained when it was on the side directed toward the source. In this case, the particles must have penetrated to about half the diameter of the organism or approximately 20 μ. While carrying out some work on eggs of the sea urchin, *Arbacia punctulata,* we also have obtained some idea of the penetration of alpha particles into protoplasm.

Arbacia eggs, when placed in a hanging drop, settle to the lower surface where they can be irradiated with alpha particles. A sample of eggs exposed for 10–15 minutes while in the two-cell stage and examined at subsequent intervals will contain deformed individuals similar to those shown in Fig. 3, row *A*. If exposed while in the four- or eight-cell stages, figures like those in rows *B* and *C*, respectively, will be found. Normally, the cells divide equally and simultaneously during early cleavage so that the number of cells increases 2–4–8–16, etc. It appears that during exposure some of the eggs by random distribution took a position with one cell (in the first case) shielded by the other from the radiation and that the radiation penetrated only far enough to destroy the cell nearest the surface. Since the over-all diameter of this organism in the two-cell stage is about 75 μ and since alpha particles appear to have penetrated not more than half this distance, their range in this case must have been less than 40 μ.

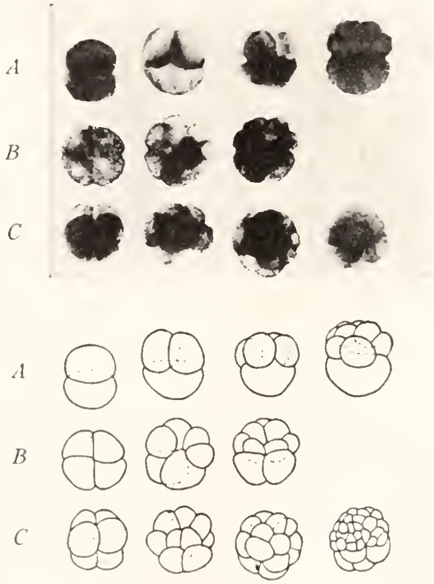

Fig. 3. Photomicrographs and diagrams showing the effects of alpha particles on *Arbacia* eggs when exposed in the 2-cell (*A*), 4-cell (*B*), and 8-cell (*C*) stages.

From the instances cited, it seems clear that the range of alpha particles in protoplasm is approximately 30 μ. Since then, the transverse diameter of the *Drosophila* egg is about 100 μ, it is improbable that the particles ever reach their center. In the case of the experiment above they were probably stopped near the surface since they passed through 2 cm. of air and the membranous chorion before reaching the living parts. Since the difference in results obtained with the different radiations may be accounted for on the basis of penetration (see below), other properties of the radiations need not be considered.

The tests performed to make certain that alpha particles and not some other radiation were responsible for the results obtained depended on differences in penetration range of the different radiations and may be given briefly at this point. An ordinary piece of writing paper was placed over the polonium-plated disc during exposure in one case, and the eggs were placed at a distance of 4 cm. from the disc in another. In both cases no eggs were killed, although the time of exposure was continuous from the end of collection to the time of hatching. Since the beta and gamma rays which might be present would have a greater range than 4 cm. of air and would not be stopped by a sheet of paper, the observed effects may safely be attributed to alpha particles.

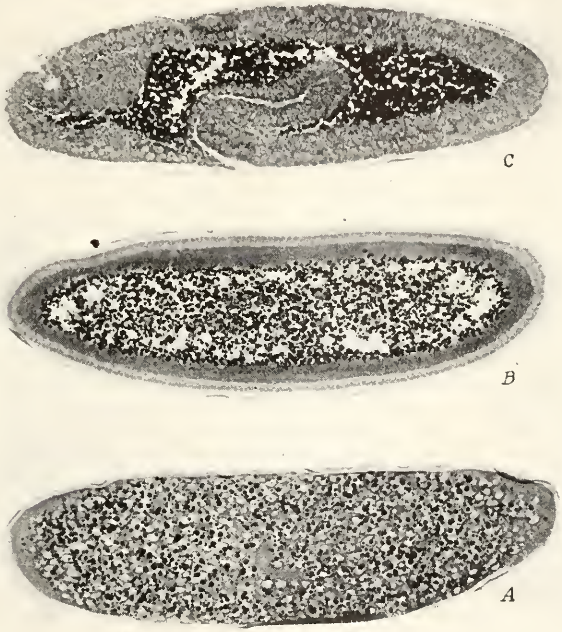

Fig. 4. Longitudinal sections of *Drosophila* eggs of different ages: (*A*) before nuclei begin to migrate to periphery, 0–1.5 hours old after fertilization (*B*) blastular stage, 1.5–2.5 hours after fertilization; and (*C*) gastrular stage, 2–3 hours after fertilization.

EARLY EMBRYOLOGY IN DROSOPHILA

Development as it pertains to the present discussion may be described briefly. The egg and sperm nuclei unite near the center of the egg and the early cleavages, nuclear only, take place synchronously in the central region at the rate of 1 in 10–12 minutes at room temperature (22–25° C.). At about the eighth or ninth cleavage (Huettner, 1923), approximately 100 minutes after fertilization, the nuclei begin to migrate to the periphery where cell membranes are formed around them and where they arrange themselves in a single layer to form the blastoderm. This thickens by continued mitosis and growth and at about 150 minutes after fertilization gastrulation begins by invagination (Figs. 1 and 4).

CORRELATIONS

By correlating the changes in sensitivity with the location of the cells at different stages, it is seen that the eggs become increasingly susceptible as the nuclei move toward the periphery, that they are the most susceptible when the largest number of cells are active at the periphery, and that they become more resistant as the actively growing regions move away from the surface toward the center again. Because of this it is believed that the changes in susceptibility to alpha particles may be accounted for by saying that (1) the amount of radiation used is sufficient to produce death only when active cells (or nuclei) are affected; that (2) at first, the cells, being in the central region of the egg, are beyond the range of the particles; that (3) they move into the range as they pass to the periphery; and that (4) the active cells move out of the range again at the time of gastrulation.

SUMMARY

1. Changes in the susceptibility of *Drosophila* eggs to different kinds of radiation have been followed during early development.

2. It was found that the eggs become more susceptible to alpha particles during cleavage and blastulation and more resistant during organogenesis, the point of maximum sensitivity being reached at or just after the beginning of gastrulation.

3. A comparison of these findings with those obtained for 200 kv. X-rays showed that the eggs were most resistant to the X-rays when they were most susceptible to the alpha particles.

4. Further experiments with 40 kv. X-rays and gamma rays indicated that the organisms were consistent in their responses to radiation capable of penetrating uniformly to all parts.

5. It appears, therefore, that the difference in response to alpha particles was due mainly to two factors: (*a*) the short penetration range of the radiation into protoplasm, and (*b*) the movement of the active cells into and out of its range.

Acknowledgments: The writers wish to express their indebtedness to Dr. G. Failla for suggesting the use of alpha particles and for his aid and helpful suggestions during the course of the work.

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ACTION OF ELECTROLYTES ON THE DORSAL MEDIAN NERVE CORD OF THE LIMULUS HEART

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The neurogenic origin of the heart beat of *Limulus* has been demonstrated by Carlson (1909) and confirmed by the more recent work of Garrey (1930) and Samojloff (1930). This demonstration depends upon the fact that the dorsal median nerve cord, where the rhythm originates, can be separated almost entirely from the heart musculature without interrupting their connection, so that experiments can be performed on the nerve and on the musculature separately. Although the literature on the effect of electrolytes on the vertebrate heart and on certain types of invertebrate heart has been very extensive since Ringer's time, relatively few papers have appeared (chiefly by Carlson himself) on the actions of salts on the *Limulus* heart. It seemed, therefore, highly desirable that further work of this kind should be carried out, especially in reference to the action of salts on the automatic nervous rhythm.

The present work was done on the nerve only. The dorsal median nerve cord (or ganglion) was isolated from the posterior end of the heart, remaining attached anteriorly, and the heart was sectioned after the second segment. This preparation, first used by Carlson (1906), is similar to a nerve-muscle preparation. The regular contraction of the heart depends upon the rhythmic nervous discharge, originating in the ganglion; and the amplitude of contraction depends upon the intensity of the nerve impulse; presumably this depends upon the number of nerve cells acting together. The first two segments of the heart were mounted in Ringer's solution and attached to a light heart lever for graphic registration with the nerve hanging down into the test solution. Any such solution could be quickly changed for another without disturbing the preparation.

Instead of using sea water as an indifferent medium for the ganglion, a simplified medium similar in composition to Ringer's solution and isotonic with the Woods Hole sea water ($\Delta = 1^\circ.82$) was used. This modified Ringer's solution consisted of 100 vols. 0.52 M NaCl, 2 vols. 0.52 M KCl, and 15 vols. 0.29 M $CaCl_2$, and proved highly satisfactory as a substitute for normal sea water or *Limulus* plasma. In this medium both the nerve and the whole heart could be kept in good condition for several hours, although in some cases there was a slight temporary increase in rate during the first few minutes after transfer from sea water to the medium. The hydrogen-ion concentration of this solution was slightly on the acid side of neutrality (pH ca. 6); the same was true of the other salt solutions used in this work. Any effect due to the change of hydrogen-ion concentration was inappreciable in these experiments, except in a few cases to be described later.

Isotonic solutions were used throughout. When the salt concentration, considered by itself, was lower than isotonic, the solution as a whole was made isotonic by the addition of sucrose. According to Garrey's determinations (1915), 0.52 M NaCl, 0.29 M $MgCl_2$, and 0.73 M sucrose are isotonic with the Woods Hole sea water, and hence with the Ringer's solution and the blood. All experiments were done at room temperature; this varied from 21° to 27° C. The action of a given salt solution was indicated by the change of activity observed in the heart muscle when the Ringer's solution surrounding the ganglion was exchanged for the experimental solution at the same temperature. All solutions were in gaseous equilibrium with the air and uniform in pH.

THE ACTION OF SINGLE SALTS

Neutral Sodium Salts

As shown by Carlson (1906*a*), the first effect of pure isotonic NaCl solution is to increase the rate of the nervous rhythm; this effect is followed by decrease in the intensity of the nervous discharge, as shown by lessened height of contraction, irregularity of rhythm, and ultimate standstill. The time for the abolition of the rhythm varies from half an hour to an hour or more. The height of contraction may increase somewhat at the beginning or may not. The invariable effects are (1) the rapid rate, (2) the irregularity of the rhythm and amplitude and (3) the gradual decrease and final cessation of contractions. All the other neutral sodium salt solutions have similar actions, differing only in degree. The quantitative differences between the neutral sodium salts follow the anion series of Hofmeister (lyotropic series); this can be demonstrated in several ways, as follows:

(*a*) By the relative effectiveness in increasing the rate of the nervous rhythm. When a nerve is transferred from Ringer's solution to a solution of pure NaCNS, for instance, the rate (contractions per minute) is only slightly increased, but the height of contraction falls off rapidly and the rhythm ceases in a minute or two. NaI has exactly the same action, except that the rate of increase is greater, and the rhythm is maintained longer, usually for two to three minutes. A specific toxic or inhibitory effect of the pure Na-salts is thus shown, for when all electrolytes are removed by surrounding the ganglion with isotonic sucrose solution, the rhythm persists for some time, frequently for half an hour or more. The relative effectiveness of the different salts in increasing the rate shows the order of the Hofmeister series, NaCNS being the least and Na_2SO_4 the most effective. Table I and Fig. 1 show the typical effect on the rhythm in the different solutions during the first five minutes after transfer from Ringer's solution, expressed as percentages of the original rates in Ringer's solution.

The data in Table I were obtained with a single heart The ganglion was first treated with NaCNS solution until the rhythm ceased completely; the NaCNS was then replaced by Ringer's solution; rhythm, at first slow, was resumed, becoming normal in rate in a few minutes. The Ringer's solution was then replaced by the second solution in the series (NaI), and the beats were counted as before until cessation. Rhythm was again restored by return to Ringer's solution as before, and the next salt solution was used similarly, and so on in the order indicated in Table I. The series was then repeated with the same solutions in the reverse order; Ringer's solution being first replaced by Na_2SO_4, then by NaCl and so on. The effect of treatment for five minutes with the Na-salt solution was always perfectly reversible; in every case normal rhythm returned after transfer to Ringer's solution. The ganglion was apparently uninjured at the end of the series. Each figure in Table I thus represents the average of two determinations on the same ganglion.

Table I

Relative effectiveness of the neutral sodium salts in increasing the rate of rhythm. Numbers represent percentage increases in rate over the control, *i.e.:*

$$\left(\frac{\text{rate in test solution} - \text{rate in Ringer}}{\text{rate in Ringer}} \times 100\right)$$

	1st min.	2d min.	3d min.	4th min.	5th min.
NaCNS..........	18	stopped			
NaI..............	72	77	stopped		
NaBr............	54	84	124	147	155
$NaNO_3$...........	82	145	165	170	160
NaCl.............	96	129	170	205	235
Na_2SO_4...........	210	305	480		

The relative effectiveness of the different salts in accelerating the rhythm shows the order:

$$Na_2SO_4 > NaCl > NaNO_3 > NaBr > NaI > NaCNS.$$

It is remarkable that, although NaCNS is the least effective in accelerating the rhythm, it is the most effective in causing standstill; *i.e.*, the order of relative toxic action of these salts is the reverse of the order of effectiveness in increasing the rhythm (with the apparent exception of Na_2SO_4, which differs from the others in being a calcium-precipitating salt). The same order of relative toxicity is also found with ciliary movement, another form of automatic rhythmic activity (Lillie, 1906, 1909; Höber, 1909), with spermatozoa and eggs (Gellhorn, 1927 *a* and *b*), and with many other cells and tissues (Höber, 1926). In general, therefore, the order of toxicity of the series of Na-salts on *Limulus* nerve agrees very well with that observed in other tissues, as well as with the order of relative action on colloidal systems.

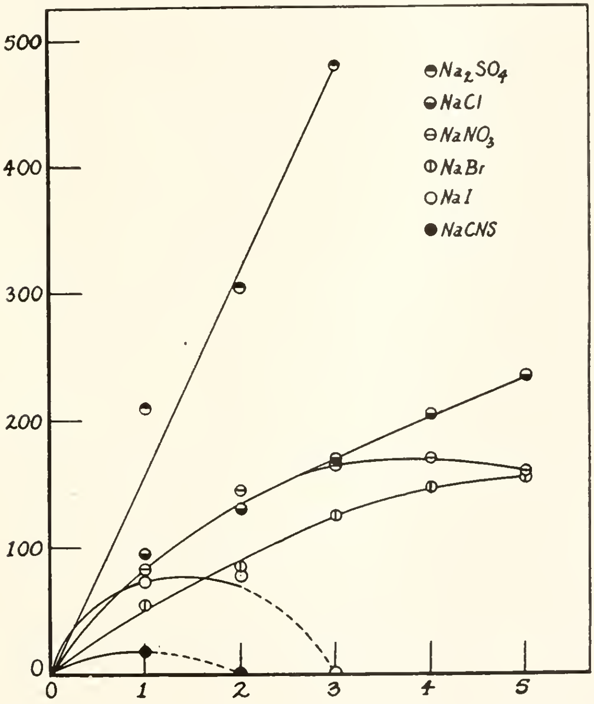

FIG. 1. Relative effectiveness of the neutral sodium salts in increasing the rate. Ordinate: percentage of increase in rate. Abscissa: time in minutes.

(*b*) The same order of relative toxicity is also shown when the NaCl in Ringer's solution is replaced by equivalent amounts of the other Na-salts. In such a modified Ringer's solution the ganglion shows a decline in activity, which is well marked at the end of ten minutes or sooner and varies with the nature of the substituting salt. In Table II the effects observed in a typical series are summarized.

All the Na-salts, except Na_2SO_4, on replacing NaCl in Ringer's solution, decrease both rate and amplitude, and the order of relative effectiveness again follows the Hofmeister series. Na_2SO_4 alone increases the rate, apparently because of its calcium-precipitating property. Calcium sulphate was actually seen to be precipitated in the solution. This decrease in calcium would naturally remove part of its antagonistic effect and increase the rate (see below).

In the frog's heart the replacement of NaCl in Ringer's solution by other Na-salts gives a similar series, but the order of relative action is reversed. While Na-salts of CNS, I, Br, and NO_3 increase the amplitude, frequency, and pulse volume, Na_2SO_4 decreases them (Handovsky, 1923) (Sakai, 1914). On the theory that the quantitative differences in the physiological effects of the Na-salts are to be referred to their differences of action on the colloidal structures of the living tissue (Höber, 1926), it would appear that the colloidal structures directly affected by the salts differ in the *Limulus* nerve and in the vertebrate heart in such a way that the orders of relative action are opposed. Differences of this kind are not uncommon in living tissues (see below).

TABLE II

Changes in rate and amplitude on replacing NaCl in Ringer's solution by other Na-salts. Numbers give the percentage change of rate for 10 minutes as compared with normal rate in Ringer's solution.

	Percentage change of rate	Amplitude
Na_2SO_4	+30	gradually decreasing, about 2/3 normal at end of 10 minutes
NaBr	−18	slight decrease but regular
$NaNO_3$	−34	more decrease and less regular
NaI	−37	rapid decrease and irregular
NaCNS	−46	decrease more rapid and more irregular

(*c*) The same series is also shown in *Limulus* nerve when we compare the relative effectiveness of the salts in initiating new rhythm after immersing the ganglion for varying lengths of time in solutions of $CaCl_2$, KCl, non-electrolyte, or in other unbalanced media which abolish the rhythm. To produce this effect isotonic $CaCl_2$ was found to be the most convenient solution, since this salt is less toxic than KCl and is more rapid in its effect than a non-electrolyte, such as sugar. When a nerve is transferred from Ringer's solution to pure isotonic $CaCl_2$, the rhythm ceases in about one minute. If the nerve be then transferred to a Na-salt solution, new rhythm is initiated sooner or later, the interval depending upon the time of immersion in $CaCl_2$ and the nature of the Na-salt. The results of a typical series of such experiments are seen in Table III, in which are given the intervals required for the reappearance of the rhythm in the isotonic solutions of the various Na-salts after inhibiting the ganglion by immersion in isotonic $CaCl_2$ for one to three minutes.

The relative effectiveness in initiating new rhythm follows the order

$$Na_2SO_4 > NaCl > NaNO_3 > NaBr > NaI > NaCNS.$$

TABLE III

Intervals after which new rhythm appears in isotonic solutions of neutral Na-salts after inhibition by immersion in isotonic $CaCl_2$ for varying lengths of time.

	Duration of $CaCl_2$ treatment		
	1 minute	2 minutes	3 minutes
sea water...			immediate recovery
Na_2SO_4.....			less than 50 sec.
NaCl.......		less than 30 sec.	after 55 sec.
$NaNO_3$.....		after 35 sec.	after 2 min. and 15 sec.
NaBr......		end of 4th min.	after 3 min.
NaI........	35–60 sec.		no recovery in 5 min.
NaCNS....	end of 5th min.		no recovery in 5 min.

A balanced solution like sea water is more effective than any pure Na-salt solution.[1]

(*d*) It was shown by Schwarz (1907) and later by Gellhorn (1932, *a* and *b*) that if a frog's skeletal muscle be fatigued to loss of irritability by repeated stimulation while immersed in a solution of a given Na-salt (diluted with isotonic sucrose or Ringer's solution), it will recover irritability when transferred to pure solutions of certain other salts but not in all, the possibility of recovery depending upon the position of the salt in the Hofmeister series. If, for example, a muscle be fatigued in

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[1] For restoring the rhythm in the frog's heart after standstill in isotonic sugar solution, Gellhorn (1932*a*) found NaCNS to be the most effective salt, the other Na-salts showing the lyotropic order of relative effectiveness but in the reverse order to that found in *Limulus* nerve.

$NaNO_3$, it will recover in a solution of any salt on the right of $NaNO_3$ in the series

$$Na_2SO_4, NaCl, NaNO_3, NaBr, NaI, NaCNS$$

but not in solutions of the salts on the left.

Similarly, when the rhythmic activity of a *Limulus* ganglion has ceased in a given pure Na-salt solution, new rhythm may, or may not, be restored on transfer to another Na-salt solution, according to the effectiveness of the salt in initiating new rhythm as found above. When the rhythmic nervous discharge has stopped in NaI solution, for example, new rhythm is initiated by NaBr or any other salt on the left of NaI in the above series, but not by NaCNS. If the rhythm is stopped in NaCNS solution, new rhythm is initiated by NaI and all the other salts. When the rhythm is stopped by $NaNO_3$, however, only NaCl and Na_2SO_4 can initiate the new rhythm. This kind of experiment is best performed by abolishing the rhythm with NaCNS and NaI and then observing the recovery in the other salt solutions. For the other salts the rhythm is abolished only after a long time, and by this time the nerve is usually in bad condition. In such cases it is difficult to repeat the process of inhibition and recovery on the same nerve; for this reason the whole series has not been systematically investigated. The experiments so far performed have, however, shown uniformly that when a nerve has lost its rhythmic activity in a given salt solution, no recovery occurs in any other solution that is less effective than itself in initiating new rhythm. Here again the order of relative activity is the reverse of that found in the vertebrate tissue; some difference in the electrical properties of the structural colloids in the two cases is indicated.

In general we conclude that the effect of all pure Na-salt solutions on the activity of the ganglion is twofold: (1) a stimulating effect shown in the initiation of new rhythm and acceleration of the normal rhythm, and (2) a toxic effect shown in the decrease of amplitude of contraction and eventual standstill. The initiation and maintenance of rhythm are an important function of the Na-salts; no other salts, except Li-salts (see below), nor non-electrolytes can initiate new rhythm. Similarly, NaCl is a stimulating agent for the vertebrate heart in general (Lingle, 1900), for many invertebrate hearts and for the frog's lymph heart (Moore, 1901). It also can produce rhythmical stimulation in vertebrate muscle (Loeb, 1899) and nerve (Mathews, 1904), and it is equally important in the maintenance of the irritability of these tissues (Overton, 1902, 1904).

Other Sodium Salts: Sodium Acetate

This salt is not included in the series of neutral sodium salts since its solution has an alkaline reaction. In pure isotonic solution it has a pronounced stimulating effect on the nerve, increasing the rate several times in a few minutes; its efficiency in this respect is intermediate between that of Na_2SO_4 and that of NaCl. It differs from the other Na-salts in that it rapidly decreases the intensity of nervous discharge, the amplitude of contraction being only about one-seventh of the normal at the end of the third minute; recovery also is slow and incomplete, indicating the infliction of some permanent injury upon the nerve.

Calcium-precipitating Sodium Salts

In general, all the calcium-precipitating salts (sulphate, fluoride, tartrate, and citrate) show the same type of influence on the *Limulus* nerve; all produce at first a rapid rhythmical discharge, shown by irregular contractions and tetanus in the muscle, followed by cessation and muscular relaxation within one or two minutes. The stimulating effectiveness, as shown by the rapidity of the initial rhythm and the completeness of the tetanus, is greatest with citrate and least with

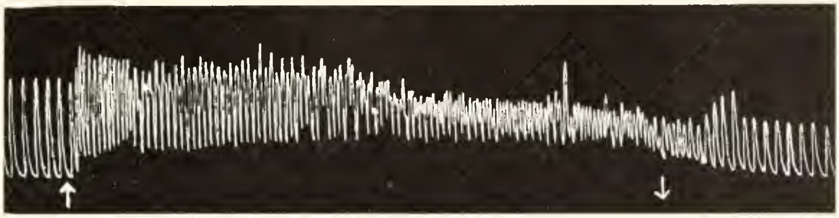

FIG. 2. Between the arrows, sea water was replaced by isotonic Na_2SO_4 for three minutes. All figures read from left to right.

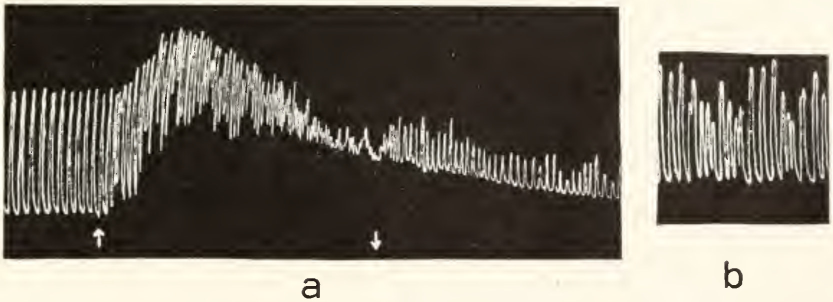

FIG. 3. (*a*) Between the arrows, Ringer's solution was replaced by isotonic sodium tartrate for two minutes.
(*b*) After 43 minutes in Ringer's solution.

sulphate; tartrate and fluoride are intermediate. The depressant or toxic effect, as measured by the time required for recovery in Ringer's solution, is greatest with tartrate; then come (in the order) fluoride, citrate, and sulphate (see Figs. 2, 3, 4, and 5).[2]

Lithium Chloride

In general LiCl has an action similar to that of NaCl, but is decidedly more toxic. The initial increase in rhythm is much greater and is followed in a few seconds by irregularity. On returning the ganglion to Ringer's solution recovery is very slow, requiring half an hour or more to restore the normal rhythm. LiCl can also initiate new rhythm but is far less effective than NaCl; typically, after treating the ganglion with $CaCl_2$ for three minutes, NaCl can initiate new rhythm in one minute, while LiCl requires at least three minutes. When a nerve that has failed to recover after three minutes in LiCl is transferred into NaCl, new rhythm starts immediately. Like the other neutral sodium salts, LiCl can not replace NaCl in Ringer's solution without harmful effect.

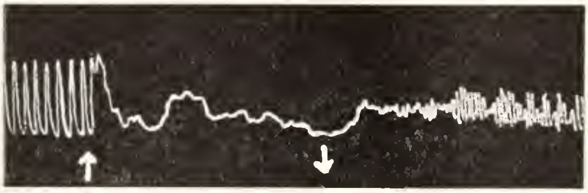

FIG. 4. Between the arrows, Ringer's solution was replaced by isotonic sodium citrate for one minute.

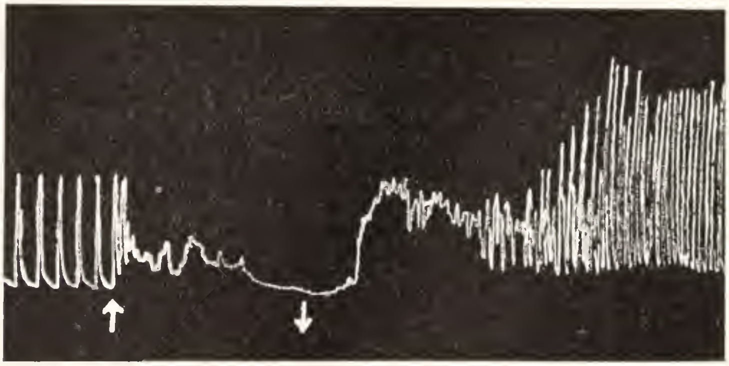

FIG. 5. Between the arrows, Ringer's solution was replaced by isotonic NaF for one minute.

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[2] The intense stimulating action of this group of calcium-precipitating salts is also seen in frog's nerve (Mathews, 1904) and in skeletal muscles of various kinds (Garrey, 1905).

Potassium Chloride

Carlson (1906*d*) found that when the ganglion is immersed in pure isotonic KCl there is an immediate increase in the height of the first one or two contractions; this is followed by a feeble but rapid rhythm and standstill in a minute or less. A primary stimulating effect and an inhibitory effect of KCl are thus distinguishable. The stimulating effect is particularly evident when KCl is present in high concentration in Ringer's solution. Inhibition by KCl is perfectly reversible if the nerve is not subjected to its action too long. Recovery in normal Ringer's solution, however, is always slower than after Ca-inhibition, and a rapid rhythm always appears at the beginning of the recovery.

Salts of Alkali Earth Metals

Of the commonly used salts of the alkali earth metal group, chlorides of Ca, Mg, and Sr have similar action with certain differences of degree. Pure isotonic $BaCl_2$, however, as first shown on skeletal muscle by Loeb (1899), on vertebrate nerve by Mathews (1904) and on the muscle and nerve of the *Limulus* heart by Carlson (1906*d*), has an intense primary stimulating effect, somewhat like that of KCl, producing a rapid initial rhythm followed by rapid inhibition. When the $CaCl_2$ in Ringer's solution is replaced by an equal amount of $BaCl_2$, the rhythm is increased in rate and becomes irregular.[3]

TABLE IV

Average change in rate for 5 minutes (expressed as percentages of the original rate in Ringer's solution) following the addition of varying amounts of $CaCl_2$, $MgCl_2$, and $SrCl_2$ to Ca-free Ringer's solution.

	Vols. of isotonic alkali earth salt solutions added to 100 vols. isotonic NaCl and 2 vols. KCl				
	0	5	15	30	50
$MgCl_2$	+82	+41	+32	+ 3	−14
$CaCl_2$		+22	0	−11	−25
$SrCl_2$		+17	−12	−21	−31

Chlorides of Ca, Mg, and Sr in pure isotonic solutions decrease both the rate and the intensity of the nervous discharge, without any indication of a primary stimulation, and arrest the rhythm in a few minutes. The order of relative effectiveness in arresting the rhythm is

$$Ca > Mg > Sr.$$

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[3] $AlCl_3$ has a similar action, causing rapid initial rhythm and rapid inhibition. Solutions of $AlCl_3$ have, however, an acid reaction.

Typically, in experiments on the same nerve, Ca arrests the rhythm in about one minute, Mg in about two minutes, and Sr within three minutes. When the salts are added in varying quantities to Ringer's solution surrounding the ganglion a retardation of rhythm is also seen, but the order of relative effectiveness is different. Table IV gives the percentage change in rate of one nerve during five minutes when the ganglion is placed in Ca-free Ringer's solution (*i.e.*, a mixture of 100 vols. 0.52 M NaCl plus 2 vols. 0.52 M KCl) to which varying proportions of the three salts are added, as compared with the control rate in normal Ringer's solution.

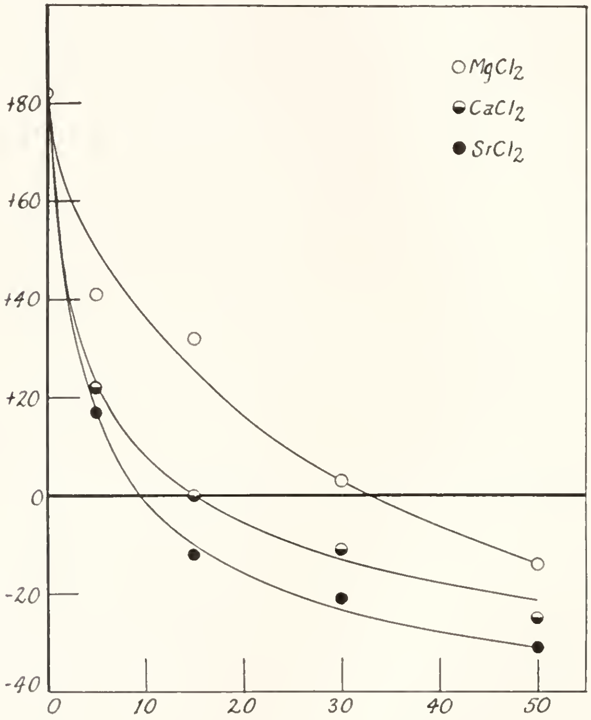

FIG. 6. Comparison of varying amounts of $MgCl_2$, $CaCl_2$, and $SrCl_2$ in Ringer's solution. Ordinate: average percentage of change in rate for five minutes. Abscissa: volumes of isotonic solutions of the alkali earth salts added to 100 vols. NaCl and 2 vols. KCl.

The calculations on the average percentage change in rate were made as follows. When the nerve was immersed in a solution of 100 vols. isotonic NaCl plus 2 vols. isotonic KCl, the rhythm increased from 17 beats per minute in Ringer's solution to 23, 31, 32, 33, 36 in the successive minutes. The average increase in rate for the five minutes was from 17 to 31, or an increase of 82 per cent. All the calculations for percentage change in rate were made in the same way.

It is evident from Table IV and Fig. 6 that the relative effectiveness of the three cations in decreasing the rate under these conditions follows the order

$$\text{Sr} > \text{Ca} > \text{Mg}.$$

Kisch (1927*b*) found the same series in a study of the influence of these salts on the frequency of the vertebrate heart.

It might be expected that for each of the three salts a certain concentration could be found at which the normal rhythm would be maintained as in Ringer's solution. According to the curves of Fig. 6, about thirty volumes of $MgCl_2$, or ten volumes of $SrCl_2$, should have the same effect as the 15 volumes of $CaCl_2$. This is, however, not exactly the case. For example, in the mixture of 30 vols. $MgCl_2$, 100 vols. NaCl, and 2 vols. KCl there was actually a slight increase in rate for the first two minutes, followed by a continuous gradual decrease to a value below normal. Evidently $MgCl_2$ by itself is unable to make a balanced solution with NaCl and KCl in spite of the fact that magnesium is normally more concentrated in sea water than calcium. If $MgCl_2$ is used together with $CaCl_2$ in the normal proportion of sea water (Mg/Ca *ca.* 3), the normal rhythm is maintained quite well. This inability of $MgCl_2$ to replace $CaCl_2$ in Ringer's solution was also observed by Kisch (1929) and by Goljachowski (1932), using the vertebrate heart. In the *Limulus* ganglion $SrCl_2$ was more favorable than $MgCl_2$ as a substitute for $CaCl_2$ in Ringer's solution; yet even under the best conditions a gradual decrease in rate was also found. Apparently neither $MgCl_2$ nor $SrCl_2$ can completely replace $CaCl_2$ in Ringer's solution.

SPECIFIC RÔLES OF NaCL, CaCL$_2$, AND KCL IN RINGER'S SOLUTION AND THEIR ANTAGONISM

As we have seen, NaCl appears necessary for the maintenance of the ganglionic rhythm; but in pure isotonic solution of this salt the rhythm is rapid and irregular and cannot be long maintained. When to this solution $CaCl_2$ alone is added to a concentration equal to that present in normal Ringer's solution, the rhythm becomes regular and is greatly reduced in rate, although still remaining above normal, while the amplitude of the contraction is increased somewhat. But even so the normal rhythm cannot be maintained, and the amplitude gradually decreases. It is only after the addition of the normal content of KCl in Ringer's solution that the medium becomes capable of maintaining the normal rhythm for a long time (see Fig. 7). A nerve can be kept in good condition for more than six hours in this balanced solution with

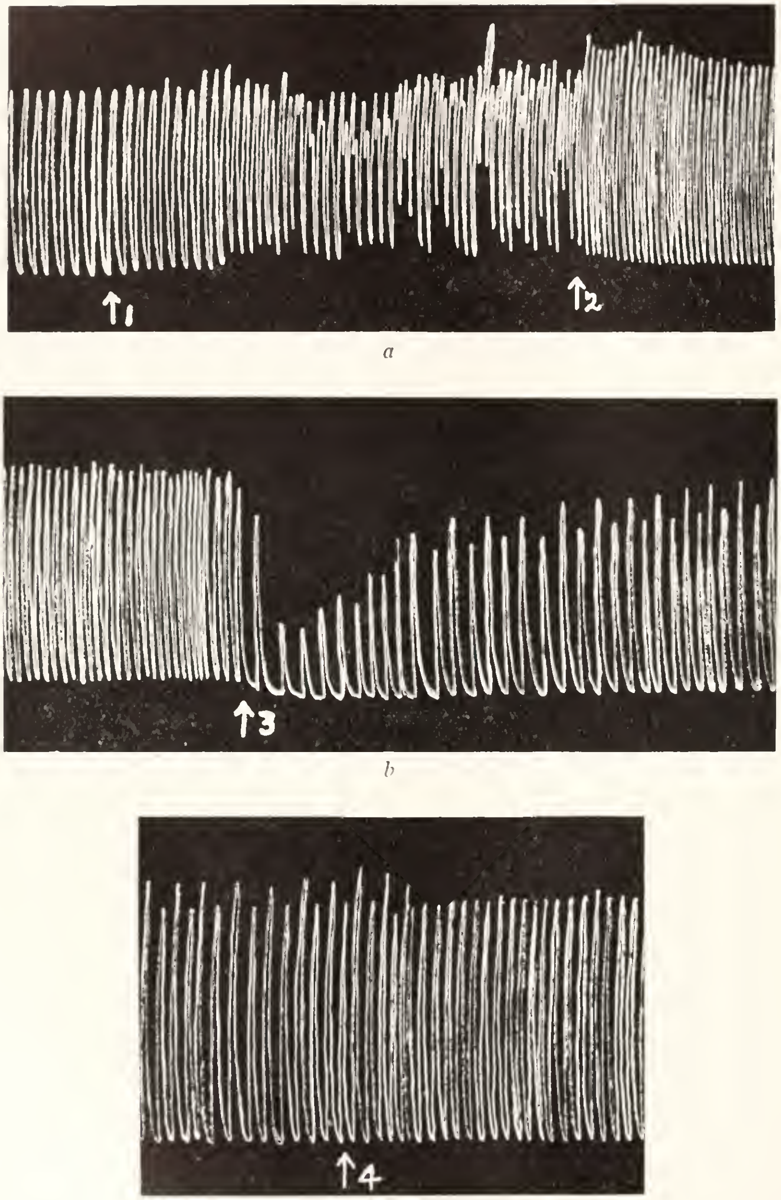

FIG. 7. Effect of transferring a ganglion from Ringer's solution.

(*a*) To pure isotonic NaCl at 1 for two minutes, then to a mixture of 100 vols. NaCl plus 30 vols. $CaCl_2$ at 2.

(*b*) Three minutes later, to a mixture of 100 vols. NaCl plus 30 vols. $CaCl_2$ and 4 vols. KCl at 3, showing the potassium-paradox.

(*c*) Nine minutes later, back to Ringer's solution again at 4.

only a very gradual decrease in the rate and amplitude of the heart beat. It seems, therefore, that although $CaCl_2$ antagonizes part of the toxic effect of pure NaCl the antagonism is by no means complete. Apparently the remaining part of the total effect of the NaCl is antagonized by KCl. Whether or not KCl antagonizes $CaCl_2$ in normal Ringer's solution then becomes a question. It will, however, be shown below that there is no definite antagonism between K and Ca at their normal concentration in Ringer's solution. Working with marine organisms, Rubinstein (1926, 1927) has come to a similar conclusion; namely, that NaCl has two different toxic actions, one antagonized by K and the other antagonized by Ca, and that in the ordinary balanced solution there is no antagonism between K and Ca.

A striking phenomenon is observed when a nerve is transferred from the solution containing only NaCl and $CaCl_2$ to the normal Ringer's solution containing also KCl. Instead of returning to its normal rhythm at once, either the rate or the amplitude (or both) is still further decreased, or in some cases the rhythm may cease altogether, and then after an interval return gradually to normal. This peculiar phenomenon has been described in the case of the frog's heart by Libbrecht (1920, 1921) and is called by him the potassium-paradox. It is obtained when the potassium-free perfusion fluid is exchanged for normal Ringer's solution, and is believed to be purely a potassium effect. Working on the rabbit, Busquet (1922, *a* and *b*) came to the opinion that the potassium-paradox is not a direct potassium effect but is connected with vagus stimulation. This idea has been refuted by Kisch (1927*a*). The production of potassium-paradox on the *Limulus* nerve also shows its independence of vagus action. The work on the vertebrate heart has shown that the effect depends on several factors: (*a*) on the difference in the potassium content of the two solutions (Libbrecht, 1921) (Witanowski, 1926), (*b*) on the duration of the perfusion with the K-free or K-poor solution (Kisch, 1927*a*), and (*c*) on the presence of a certain proportion of Ca (Kisch, 1927*a*). In general, the conditions are similar in the *Limulus* ganglion. The same type of potassium-paradox effect is obtained not only on changing from a K-free solution to Ringer's solution but also on changing from Ringer's solution to a solution of higher K-content. It is more readily obtained after a prolonged exposure to the K-free solution, or after repeated immersion in it. It is not obtained on changing from a pure isotonic sucrose solution to a sucrose solution containing KCl in its normal concentration in Ringer's solution, indicating that the presence of some other electrolytes is necessary. The exact conditions under which the K-paradox can be produced in the *Limulus* nerve have not been worked out in detail. It is interesting to note that Kisch (1930) has also found a similar phe-

TABLE V

Average change in rate for 5 minutes (in percentage of control rate) on addition of NaCl to 2 vols. KCl plus 15 vols. $CaCl_2$.

Vols. NaCl added	200	150	100	50	10	0
Per cent rate change	+96	+30	0	−8	−27	−36 *

* Average rate for the last two minutes of rhythm (beat becomes inappreciable in five minutes).

nomenon with Ca and Sr (Ca- and Sr-paradox). The general nature of the paradox suggests that the deficiency of a certain ion (*e.g.* K-ion) produces a change in the tissue of such a kind that the subsequent addition of a small amount of the ion gives rise to its specific effect in an exaggerated form.

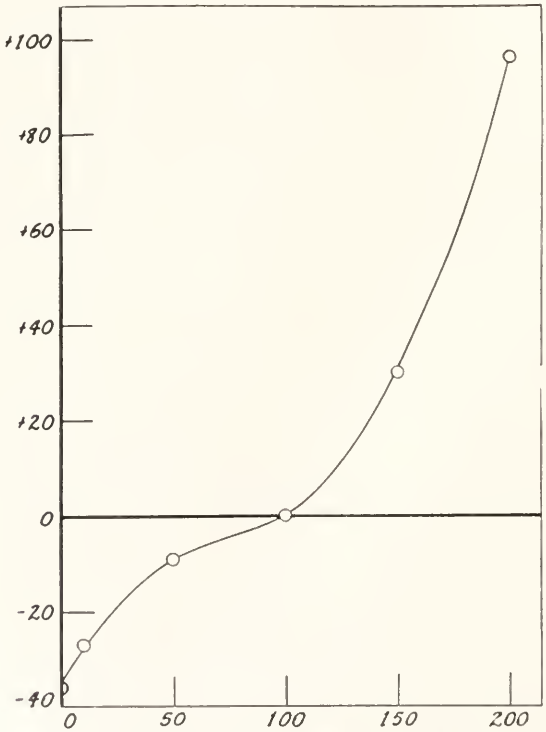

FIG. 8. Effect of varying the amount of NaCl in Ringer's solution. Ordinate: average percentage of change in rate for 15 minutes. Abscissa: vols. of isotonic NaCl added to 15 vols. $CaCl_2$ and 2 vols. KCl.

The specific rôles of the three salts in Ringer's solution can be studied by varying the concentration of each of them separately.

Effect of Varying the NaCl Content of Ringer's Solution

The normal Ringer's solution for *Limulus* heart contains 100 vols. 0.52 M NaCl, 15 vols. 0.29 M $CaCl_2$, and 2 vols. 0.52 M KCl. If, keeping constant this ratio of $CaCl_2$ and KCl, one increases the relative concentration of NaCl, a rapid rate and some irregularity appear, characteristic of the action of unbalanced NaCl. When NaCl is present in a smaller ratio than 100 vols., the rhythm is retarded, showing the effect of excess of Ca and K. Table V and Fig. 8 show the effect of varying the relative concentration of NaCl in Ringer's solution.

Effect of Varying the $CaCl_2$ Content of Ringer's Solution

In general, increase in the proportion of Ca in Ringer's solution decreases the rate of beat. Table VI gives the average results of three experiments.

The curves in Fig. 9 show the antagonism between NaCl and $CaCl_2$ beyond any doubt. As regards NaCl and $CaCl_2$, it is found that when the ratio of Ca : Na is less than 0.28 the rate is increased, indicating the predominance in the action of NaCl. When the ratio is greater than 0.28 the rate is decreased, showing a predominance of $CaCl_2$. No mixture containing only NaCl and $CaCl_2$ can maintain the normal rhythm for long; a certain amount of KCl is indispensable. This is also shown by the lower curve in Fig. 9, giving the effect of the addition of $CaCl_2$ to a mixture of 100 vols. NaCl and 2 vols. KCl. When the proportion of $CaCl_2$ is less than 15 vols. the rate is increased; with more than 15 vols. it is decreased. The parallel course of the two curves is remarkable, showing a nearly constant influence of the KCl. There is no indication of any direct antagonism between Ca and K. Each of these cations appears to antagonize its own fraction of the toxic effect of pure NaCl, quite independently of the other.

TABLE VI

Percentage of change in rate for 5 minutes on addition of $CaCl_2$ (1) to 100 vols. NaCl and (2) to 100 vols. NaCl plus 2 vols. KCl.

Vols. $CaCl_2$ added	(1) to 100 vols. NaCl	(2) to 100 vols. NaCl and 2 vols. KCl	(1) − (2)
0	+123	+92	31
15	+ 27	0	27
30	− 5	−25	20
50	− 30	−46	16

Effect of Varying the KCl Content of Ringer's Solution

As mentioned above, in a solution containing 2 vols. 0.52 M KCl plus 100 vols. 0.52 M NaCl the rate is slower than in pure NaCl. If more KCl is added, the rate begins to increase again (upper curve, Fig. 10). A curve of similar shape is obtained on the addition of KCl to a mixture of 100 vols. NaCl and 15 vols. $CaCl_2$ (lower curve, Fig. 10). Changing the KCl content of Ringer's solution in either direction (above or below the normal concentration of K) always results in an increase of rate. When the KCl content is decreased below normal, the rapid rate is due to the excess of NaCl; when the KCl is increased above normal, the acceleration results from the direct stimulating action of the K-ions. Accordingly we found that the toxic effect of the pure NaCl solution can be partly antagonized by the addition of 2 vols. KCl to 100 vols NaCl; while if more KCl is added its own stimulating effect is manifested. The effects are found in the presence of the normal amount of $CaCl_2$ in Ringer's solution, indicating again the independent antagonistic actions of Ca and K in relation to NaCl (lower curve, Fig. 10). The differences of rate corresponding to the points on the two curves are seen in column four of Table VII, showing the nearly constant effect of addition of 15 vols. $CaCl_2$ to each of the solutions in column three (i.e., the

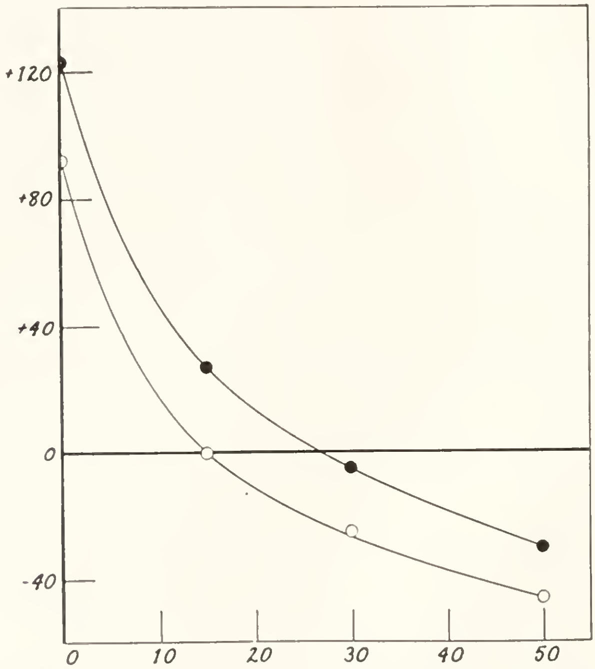

FIG. 9. Effect of $CaCl_2$. Ordinate: average percentage of change in rate for 5 minutes. Abscissa: vols. of $CaCl_2$ added (1) to 100 vols. NaCl (upper curve) and (2) to 100 vols. NaCl plus 2 vols. KCl (lower curve).

addition of a definite quantity of KCl has the same influence on the rhythm, whether it is added to the pure NaCl or to the Ca-containing NaCl solution).

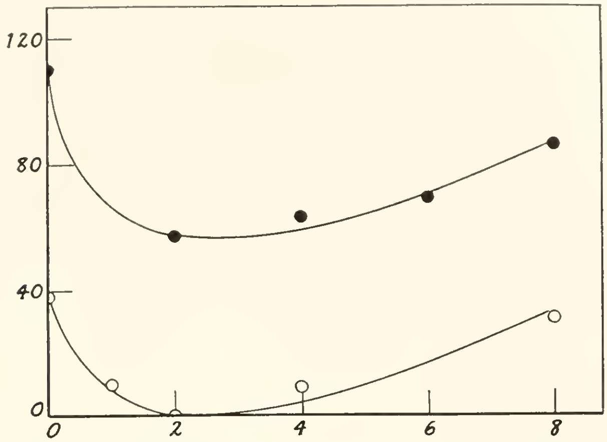

FIG. 10. Effect of KCl. Ordinate: average percentage of change in rate for 5 minutes. Abscissa: vols. KCl added (1) to 100 vols. NaCl (upper curve) and (2) to 100 vols. NaCl plus 15 vols. $CaCl_2$ (lower curve).

Effect of Varying KCl and $CaCl_2$ Together

Since an excess of KCl in Ringer's solution increases the rate and an excess of $CaCl_2$ decreases it, when both salts are present in excess we should expect to find typical antagonism. This may be tested experimentally by adding a certain definite excess of KCl to Ringer's solution; varying additions of $CaCl_2$ are then made to this solution and the resulting changes in rate during the first five minutes are deter-

TABLE VII

Percentage of change in rate for 5 minutes on addition of KCl (1) to 100 vols. NaCl and (2) to 100 vols. NaCl plus 15 vols. $CaCl_2$. Observations made on different nerves.

Vols. KCl added	(1) to 100 vols. NaCl	(2) to 100 vols. NaCl and 15 vols. $CaCl_2$	(1) − (2)
0	110	38	72
1	68 *	10	58
2	57	0	57
4	63	9	54
6	69	18 *	51
8	86	31	55

* Found from the curves in Fig. 10.

mined. When the action of K overbalances that of Ca the rate increases; when the Ca effect predominates the rate decreases. Table VIII shows the results of a series of such experiments obtained on three nerves.

TABLE VIII

Average percentage change in rate for 5 minutes on addition of $CaCl_2$ to Ringer's solution containing varying additions of KCl.

Vols. KCl added to 100 vols. Ringer	Vols. $CaCl_2$ added to 100 vols. Ringer								
	0	10	20	30	40	50	60	70	80
3	+22	− 2	−16						
6	+38	+15	+ 2	−8	−16				
9			+18	+5	− 5	−11			
12					+10	0	− 9		
18							+14	+4	−4

If the average changes in rate are plotted against the addition of $CaCl_2$, a characteristic group of curves is obtained (Fig. 11). For every concentration of KCl there should be a corresponding concentration of $CaCl_2$ at which the two salts will more or less completely antagonize each other, *i.e.* at the intersections of the curves with the abscissa. For example, in a mixture of 3 vols. KCl solution with 100

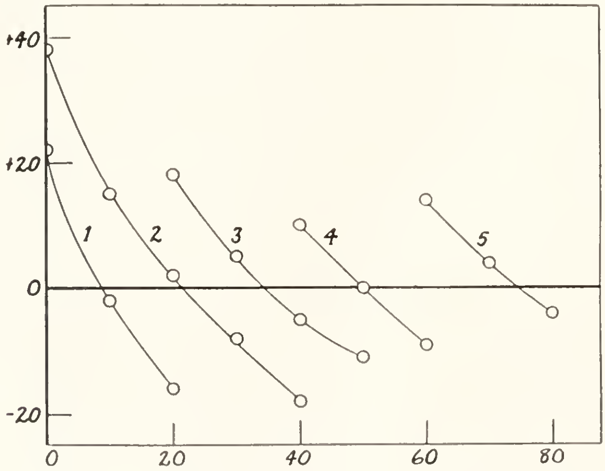

FIG. 11. Effect of addition of KCl and $CaCl_2$ together. Ordinate: average percentage of change in rate for 5 minutes. Abscissa: vols. $CaCl_2$ added to (1) 3 vols., (2) 6 vols., (3) 9 vols., (4) 12 vols. and (5) 18 vols. KCl plus 100 vols. Ringer's solution.

vols. Ringer's solution, about 9 vols. $CaCl_2$ should be added to antagonize the excess of KCl; with an addition of 6 vols. KCl, 21 vols. $CaCl_2$ should be required, and so on as represented in Table IX.

The Ca/K ratios in this table are obtained directly from the curves in Fig. 11. In general the ratio seems to increase as the absolute amounts of Ca and K become higher. If, however, the Ca/K ratio is calculated on the basis of their total concentration in Ringer's solution (by adding approximately 2 vols. to the added excess of KCl and 15 vols. to that of $CaCl_2$) the ratio becomes very nearly constant, the average being about 4.6. This indicates that Ca and K antagonize each other most completely at a definite ratio of 4.6, when they are present in excess in Ringer's solution. The minimal amounts of 15 vols. $CaCl_2$ and 2 vols. KCl are those required to antagonize the toxic effect of 100 vols. NaCl. Apparently only the excess of K is directly antagonized by the Ca (and vice versa). The value 4.6 should not be taken too strictly, for it does vary from nerve to nerve. Somewhere between 4 and 5 is a fair estimation on the whole. It should be added that the antagonism is not complete; a gradual decrease in the amplitude of contraction is always found. Evidently the three salts in Ringer's solution are so interrelated that it is only when they are present in the normal ratio that the solution forms a well balanced medium.

TABLE IX

Vols. $CaCl_2$ and KCl solutions necessary for complete antagonism of each other.

(1) added to 100 vols. Ringer			(2) added to 100 vols. NaCl		
$CaCl_2$	KCl	$CaCl_2$: KCl	$CaCl_2$	KCl	$CaCl_2$: KCl
9	3	3.0	24	5	4.8
21	6	3.5	36	8	4.5
34	9	3.8	49	11	4.5
50	12	4.2	65	14	4.6
75	18	4.2	90	20	4.5

LOSS OF RHYTHM AND LOSS OF IRRITABILITY

Minimal Concentration of Ringer's Solution for Maintenance and Initiation of Rhythm

When a nerve is immersed in pure $CaCl_2$ solution, the rhythmic nervous discharge stops in a minute or two, but the nerve can still be stimulated by a tetanizing current, as shown by the contraction of the heart. During the next several minutes (the exact time depending on the condition of the nerve) the irritability gradually declines and finally disappears completely, so that the nerve is no longer stimulated by a current of any strength. The dissociation of automatic rhythm and irritability is particularly obvious in some cases. Occasionally, when a nerve is dissected out, it may not show any rhythmic activity at all, although it always responds to the tetanizing current and to a pure NaCl solution. When a nerve with its irritability abolished in isotonic $CaCl_2$ is transferred into Ringer's solution, the rhythm appears gradually, together with the associated irritability. It is difficult to determine by experiment whether the irritability or the rhythmic activity returns first, for usually an automatic rhythm is started by any brief stimulation during the recovery period. In all cases, however, where definite rhythm is restored, the nerve responds to electrical stimulation; *i.e.*, automatic rhythm is always associated with irritability.

It was found by Overton (1902, 1904) that a minimal amount of NaCl is necessary for the maintenance of irritability in vertebrate muscle and nerve (about 20 vols. isotonic NaCl plus 80 vols. isotonic sucrose). The minimal amount of Ringer's solution necessary for the maintenance of irritability in the *Limulus* nerve has not yet been determined. A mixture of 33 vols. Ringer's solution plus 67 vols. isotonic sucrose solution can maintain the normal rhythm for more than one hour with only a slight increase in rate at the beginning followed by a slow progressive decline in rate and intensity. The initial stimulating effect is due to the specific action of sucrose (Carlson, 1906*c*). The unfavorable influence of the lowered salt concentration is clearly shown when after an immersion of one hour in the dilute Ringer's solution the ganglion is returned to Ringer's solution of normal concentration. The rate and intensity are at once increased. A solution containing 10 vols. per cent Ringer's solution in isotonic sucrose solution can not maintain the normal rhythm; both the rate and the intensity of the rhythmic impulses decrease gradually, and the rhythm stops in about twenty minutes (*i.e.*, in about the same time as in pure sucrose solution). Similarly, when the rhythm of a nerve is inhibited by $CaCl_2$, it recovers in a mixture of 30 vols. Ringer's solution plus 70 vols. isotonic sucrose. It appears, therefore, that this solution can maintain the normal rhythm of *Limulus* nerve for some time although not indefinitely, and can initiate new rhythm in a quiescent nerve.

Discussion

The precise conditions determining the rhythmical activity of the nerve cells in the *Limulus* ganglion are not clearly understood at present, but the analogy of Lillie's iron wire model (Lillie, 1929) suggests that some kind of automatic rhythmic breakdown and restoration of membrane structure in these cells is the primary physical change. The rhythmic activity is closely dependent on the environmental conditions, such as temperature (Carlson, 1906*a*), the osmotic pressure of the medium (Carlson, 1906*b*), and particularly the chemical composition of the medium. The nerve appears, however, to be comparatively indifferent to decrease of oxygen tension (Newman, 1906).

One very striking property of the ganglion is the promptness with which it reacts to the presence of electrolytes; in this respect it presents a contrast with the heart muscle. While isotonic NaCl solution stimulates the ganglion almost instantaneously, the heart from which the nerve cord has been removed develops a rhythmic series of contractions only after a prolonged immersion in the same solution, *e.g.* for 30 to 45 minutes (Carlson, 1905). In vertebrate muscle and nerve a contrast of the opposite kind is seen; the frog's sartorius shows rhythmic twitchings in isotonic NaCl solution immediately after immersion (Loeb, 1899), while under the same conditions the sciatic nerve gives rhythmic impulses only after two hours or more (Mathews, 1904). The extreme sensitiveness of the *Limulus* heart ganglion to the action of salt solutions is probably to be referred chiefly to the presence of nerve cells, although the non-myelinated structure of the fibers may also be a factor.

The action of ions on the ganglion is, on the whole, quite comparable with that observed on the vertebrate heart. Some differences in the electrical properties of the structural colloids of the tissue are, however, suggested by the reversed order of the lyotropic series, described in the first part of this paper. Another difference between the two tissues is seen in the well-established antagonism between K and Ca in the vertebrate heart (Ringer, 1882, *a* and *b*), as contrasted with the independent antagonisms of KCl and of $CaCl_2$ in reference to NaCl in the *Limulus* heart. The peculiar effect of $CaCl_2$ in arresting the vertebrate heart in systole, while with KCl the arrest is in diastole, may be attributed to special structural and chemical peculiarities of this heart. In the *Limulus* heart, every contraction is the index of a train of nerve impulses originating in the ganglion. If there is no impulse from the ganglion, the heart remains in a condition of complete relaxation. $CaCl_2$ abolishes the nerve impulse without any sign of stimulation, leaving the heart muscle in a relaxed condition.

In the foregoing description, less attention has been paid to changes in amplitude of contraction than to changes in rhythm for the reason that the former show less consistency than the latter, except in those cases cited above. The changes in rate are definite and reproducible and can be conveniently used as a criterion for the evaluation and comparison of the actions of the electrolytes. In most cases the recorded changes of rate have reference to the first five, or sometimes the first ten to fifteen, minutes after transfer from one solution to another; but these rates can not be maintained unaltered in an imperfectly balanced medium. When a nerve is immersed in an unbalanced solution for a long time, some kind of harmful effect always results and recovery is incomplete.

SUMMARY

A Ringer's solution, containing 100 vols. 0.52 M NaCl, 15 vols. 0.29 M $CaCl_2$, and 2 vols. 0.52 M KCl, has been found satisfactory as a substitute for sea water or *Limulus* blood plasma in maintaining the normal rhythmic activity of the ganglion and heart.

Neutral sodium salts in isotonic solutions exhibit (1) a stimulating effect shown by the initiation of new rhythm in a quiescent nerve and an increase in the rate of the rhythm, and (2) a toxic effect shown by the production of irregularity and early cessation of the rhythm. The relative effectiveness of the salts in initiating new rhythm and increasing the rate follows the order

$$Na_2SO_4 > NaCl > NaNO_3 > NaBr > NaI > NaCNS.$$

The toxicity follows the reverse order.

LiCl can also initiate new rhythm but is much less effective than NaCl and more toxic.

Other sodium salts, acetate, fluoride, tartrate, and citrate, have an intense stimulating action due either to their specific toxic action, or to their calcium-precipitating property, or to the alkaline reaction of some of them.

Chlorides of Ca, Mg, and Sr inhibit the rhythm with gradual decrease in rate and intensity. The relative effectiveness in decreasing the rate follows the order

$$SrCl_2 > CaCl_2 > MgCl_2.$$

$BaCl_2$ has a primary stimulating effect but inhibits the rhythm rapidly.

Pure isotonic KCl has both a primary stimulating effect and an inhibitory effect, producing a rapid rate followed by early cessation of the rhythm. At its normal concentration in Ringer's solution, it antagonizes about one-fourth of the toxic effect of the NaCl; at higher concentrations than normal it exhibits at first a stimulating or accelerating effect and later an inhibitory effect.

$CaCl_2$ at its normal concentration in Ringer's solution antagonizes about three-fourths of the toxic effect of the NaCl; at higher concen-

trations than normal it produces an inhibitory effect, decreasing the rate and amplitude.

Ca and K antagonize each other at a definite proportion (about 4 or 5 to 1) when both are present in excess in Ringer's solution.

Potassium-paradox has been observed on changing from a potassium-free solution to normal Ringer's solution.

The normal rhythm can be maintained by Ringer's solution of 30 per cent normal concentration (made isotonic by sucrose) and new rhythm can be initiated by the same solution. The rhythmic activity of the ganglion is more readily abolished than its responsiveness to electrical stimulation.

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THE EQUILIBRIUM BETWEEN HEMOGLOBIN AND OXYGEN IN THE BLOOD OF CERTAIN FISHES

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THE WOODS HOLE OCEANOGRAPHIC INSTITUTION[1]

In studies made on the hemoglobin of certain teleost fishes Root (1931) found that carbon dioxide had a remarkable effect on the ability of the blood to combine with oxygen. As in the case of mammalian blood, the addition of acid decreases the amount of oxygen combined with the hemoglobin at any given oxygen tension, but in these fish bloods the addition of acid decreases also the total amount of oxygen that can be combined even when the oxygen tension is increased to that of air. As a consequence the suggestion was made that the prosthetic groups in the hemoglobin molecule were differentially affected by the presence of acid, some being inactivated entirely so that they no longer combined with oxygen. Since confirmation of this suggestion promised to throw new light on the theory of the equilibrium between oxygen and hemoglobin, the phenomenon has been examined in greater detail.

In the present study we have examined the blood of the tautog, *Tautoga onitis* Linnæus, the goosefish, *Lophius piscatorius* Linnæus, and the toadfish, *Opsanus tau* (Linnæus). We have confirmed the observation of Root and shown that the amount of oxygen combined is decreased in the presence of acid even though the oxygen tension is raised to one atmosphere. In addition, the oxygen dissociation curves have been studied throughout the range of hydrogen ion concentration in which the phenomenon is described. These curves are remarkable as to shape, especially those of the toadfish, which have a distinct undulatory character. Another characteristic is a change in the shape of the curves with a change in pH. In alkaline solutions the curves for the goosefish and for the tautog are sigmoid whereas in more acid solutions the curves are rectangular hyperbolæ. Thus in these teleost fishes we find certain unique characteristics exhibited by the oxygen dissociation curves. Such peculiarities must be accounted for by any acceptable general theory of the union of oxygen with hemoglobins.

METHODS

The blood of the goosefish was obtained from the bulbus or sinus venosus, and was removed from the animal shortly after it was taken from the commercial fish traps. On account of the low hemoglobin concentration of the blood of this fish it was customary to centrifuge the blood and draw off plasma equal to about one-third the total volume, before making analyses. The other fishes were maintained at the laboratory in tanks, and were bled from the gills by means of a hypodermic syringe. Lithium oxalate was used as an anticoagulant. Samples of blood were necessarily combined in any given species of fish and the reserve supply kept on ice. In most cases the blood was used within 12 hours. In the case of the goosefish it was possible to keep a sample of blood several days without the appearance of methemoglobin.

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[1] Contribution No. 7.

The usual methods of obtaining oxygen dissociation curves were employed as described by Dill (1928). Carbon dioxide and oxygen analyses on the blood were done simultaneously as described by Root (1931). The analysis of gas mixtures containing over 30 per cent carbon dioxide or oxygen was made possible by a special modification of the Haldane apparatus. Gas pressures in the tonometers were calculated by use of the formula of Bock, Field, and Adair (1924). Samples of blood were equilibrated at a temperature of 25° C. for 15 minutes in a water bath. Nitrogen was used as the inactive gas in the tonometers. It is essential to point out that but one tonometer was equilibrated at a time and analyses followed directly after equilibration. This procedure is quite necessary in fish blood, since it possesses active, nucleated erythrocytes (Dill, Edwards, and Florkin, 1932). Duplicate analyses, both of the gases in the blood and in the tonometer, were made for all established points.

Since the oxygen combined by these bloods is diminished in the presence of acid, the oxygen capacities were always determined on blood to which a small amount of powdered sodium bicarbonate had been added. This blood was then equilibrated either in air or in oxygen and the figure obtained after dissolved oxygen was subtracted was taken to be the real oxygen capacity of the hemoglobin.

A few of the more acid samples turned brownish in color and the formation of methemoglobin was suspected. In such cases the oxygen capacity of a duplicate sample of the blood was determined directly after each equilibration by using sodium bicarbonate to bring the blood once more to an alkaline reaction and re-equilibrating with air. Whenever the hemoglobin had been irreversibly inactivated the percentage saturation was calculated by using the oxygen capacity as determined after equilibration rather than the original value.

All the data expressing the quantity of oxygen in blood were corrected for dissolved oxygen. In the case of toadfish and goosefish the solubility coefficient for oxygen was experimentally established as 2.70 vols. per cent for 760 mm. O_2 at 25° C. This figure was assumed to hold in the case of the tautog.

In studies of the effect of acid on the ability of blood to combine with oxygen, samples of blood were equilibrated in oxygen or air to which had been added various amounts of carbon dioxide. When acidities were desired greater than could be obtained in this way a few drops of 4 per cent lactic acid were added.

The pH of fish blood is not easy to control and great difficulties were experienced in trying to establish dissociation curves at constant pH. On account of the great change in acidity of the hemoglobin with degree of oxygenation the use of a constant carbon dioxide tension does not lead to a constant pH at all oxygen tensions. This difficulty was overcome by grading the amount of carbon dioxide added and, in some cases, by adding a small amount of sodium bicarbonate. In this way it was possible to obtain points calculated to have approximately the same pH values at all degrees of oxygenation. The pH of the blood was calculated from gasometric data, using the Henderson-Hasselbalch equation and assuming pK 6.22 for whole blood at 25° C. Dissolved carbon dioxide was calculated by using Bohr's solubility coefficient slightly modified in a manner prescribed by Peters, Bulger, and Eisenman (1924) to allow for differences in corpuscular volume. The final factors used to calculate the volume percentage of dissolved CO_2 were 0.102 pCO_2 for toadfish, and 0.1 pCO_2 for the other fish bloods. It must be remembered that the electrolyte equilibrium between cells and plasma is completely ignored and the pH is calculated from determinations of the carbon dioxide equilibrium of whole blood rather than that of either plasma or cells.

The Apparent Loss of Oxygen Capacity in Acid Solutions

Root (1931) found that the amount of oxygen combined with certain fish bloods at a pressure of oxygen equivalent to that of air depended upon the acidity of the blood. The addition of very small amounts of carbon dioxide (even 5 or 10 mm.) or of lactic acid, appreciably decreased the oxygen bound. This effect is reversible, for if the blood equilibrated under acid conditions is again made alkaline it regains its original combining capacity.

It seemed to us that this phenomenon might be merely a pronounced "Bohr" effect (Bohr, Hasselbalch, and Krogh, 1904) in which the acid altered the oxygen dissociation constant to such an extent that the oxygen tension of air was insufficient to completely saturate the hemoglobin. If higher oxygen tensions had been used, saturation might have been complete and the oxygen capacity undiminished. To test this possibility fish blood was equilibrated with oxygen containing the requisite quantity of carbon dioxide. Since the total pressure was about one atmosphere, the oxygen tension of these gas mixtures decreased with increasing carbon dioxide concentration. For this reason lactic acid was added on some of the more acid samples to enable a relatively high acidity to be reached without too greatly diluting the oxygen with carbon dioxide.

The results upon the blood of the goosefish, the tautog, and the toadfish in both air and oxygen at 25° C. are represented in Fig. 1.

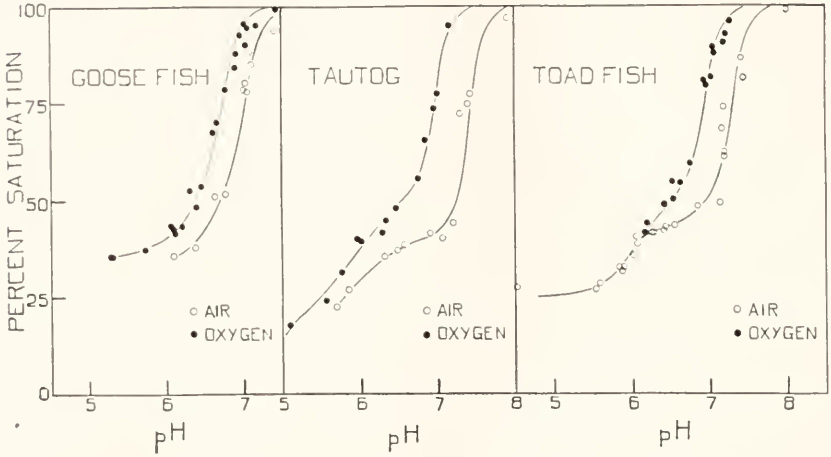

FIG. 1. The oxygen combined in fish bloods equilibrated with varying tensions of carbon dioxide and oxygen or of carbon dioxide and an oxygen tension approximating that of air.

The oxygen tension of the more acid points in the curves of the toadfish is about 500 mm., of the tautog 650 mm., and of the goosefish 350 mm. Except in the acid ranges of the toadfish blood, the samples equilibrated with oxygen combined with more oxygen than those treated with air. But it still remains a question as to whether the oxygen dissociation curves have shifted or whether the amount of oxygen which can be combined has been decreased. It seemed desirable, consequently, to determine the entire oxygen dissociation curves at various hydrogen ion concentrations in the hope that their analysis would enable us to determine the relative part played by these two phenomena.

EQUATIONS FOR OXYGEN-HEMOGLOBIN EQUILIBRIUM AT CONSTANT pH

There have been three types of equations describing oxygen dissociation which have proved useful for our purpose. The first of these is that of Hill (1910). The fraction of hemoglobin combined with oxygen, in the following manner:

$$Y = \frac{Kx^n}{1 + Kx^n}, \tag{1}$$

where x is the oxygen concentration expressed as pressure in mm. Hg. Equation (1) may be derived from the mass law on the assumption that n is the number of molecules of oxygen uniting with each molecule of hemoglobin.

According to Adair's hypothesis, each molecule of hemoglobin combines with four molecules of oxygen and the molecule $Hb(O_2)_4$ is built up and broken down in stages, thus:

$$Y = \frac{0.25K_1(O_2) + 0.5K_2(O_2)^2 + 0.75K_3(O_2)^3 + K_4(O_2)^4}{1 + K_1(O_2) + K_2(O_2)^2 + K_3(O_2)^3 + K_4(O_2)^4}, \tag{2}$$

in which K_1, K_2, K_3, and K_4 are products of mass law equilibrium constants. This equation has been successfully used to describe the curves for man (Adair, 1925), the horse (Ferry and Green, 1929), and the sheep (Ferry and Pappenheimer, 1929).

Another expression has been suggested by Redfield (Ferry and Green, 1929, p. 194) and has been found useful in describing the oxygen dissociation curves of a number of bloods containing hemocyanin which have a distinct undulatory character (Redfield, 1933). It may be assumed that the respiratory protein consists of two or more components, each of which reacts with oxygen independently of the others and reaches an equilibrium described by a distinct oxygen dissociation curve. Further, each component reacts with oxygen in accordance with Hill's equation (equation 1) but the components are each characterized by a different value of n. If the oxygen dissociation constants of the forms characterized by values of n of 1.0, 2.0, 3.0, 4.0, etc. are designated by K_1, K_2, K_3, K_4, etc., and the fraction of the total oxygen bound by each of these forms as α_1, α_2, α_3, α_4, etc., the fraction of the total respiratory protein present in the oxygenated condition, Y, is given by the equations:

$$Y = \frac{\alpha_1 K_1 x^1}{1 + K_1 x^1} + \frac{\alpha_2 K_2 x^2}{1 + K_2 x^2} + \frac{\alpha_3 K_3 x^3}{1 + K_3 x^3} + \frac{\alpha_4 K_4 x^4}{1 + K_4 x^4} \cdots$$

In applying this expression to the fish bloods which we have studied, it is not necessary to employ more than the first two terms so that the expression becomes

$$Y = \alpha_1 \frac{K_1(O_2)}{1 + K_1(O_2)} + \alpha_2 \frac{K_2(O_2)^2}{1 + K_2(O_2)^2}. \tag{3}$$

Table I

Oxygen Dissociation of Toadfish Blood Equilibrated at 25° C.

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
17	8.76	1.35	1.32	6.98	18.9	38.8	67.3	7.42
	19.4	2.57	2.50		35.8	36.3	64.7	7.44
	25.3	2.96	2.87		41.1	34.7	65.8	7.47
	44.25	3.71	3.55		50.9	36.7	67.0	7.45
	69.8	4.47	4.22		60.5	34.3	62.9	7.45
	80.0	4.62	4.34		62.2	36.1	65.1	7.44
	90.9	5.11	4.79		68.6	34.0	63.0	7.45
	107.0	5.26	4.88		70.0	35.1	64.2	7.45
	115.0	5.28	4.87		69.8	36.4	63.2	7.43
	117.5	5.30	4.88		69.9	34.9	64.0	7.45
	134.2	5.81	5.33		76.4	34.75	63.2	7.45
	151.0	6.21	5.68		81.4	33.85	61.55	7.44
	172.0	6.56	5.95		85.3	36.3	61.4	7.41
	191.0	6.72	6.04		86.6	34.1	63.5	7.44
	261.5	7.27	6.34		90.8	35.4	58.2	7.40
19 †	2.69	0.47	0.46	7.21	6.39	46.3	48.2	7.18
	14.7	1.95	1.90		26.3	45.2	47.5	7.19
	26.9	2.81	2.72		37.7	42.5	47.4	7.22
	53.8	3.41	3.22		44.7	41.4	46.35	7.22
	80.0	3.77	3.49		48.4	41.3	46.85	7.22
	102.0	4.16	3.80		52.7	42.0	46.0	7.21
	131.4	4.54	4.07		56.5	41.0	46.2	7.21
	147.0	4.94	4.42		61.3	43.0	45.1	7.19
	163.4	5.24	4.66		65.2	38.9	44.7	7.23
	209.5	5.79	5.05		70.0	43.8	44.1	7.17
	247.0	6.31	5.44		75.5	40.75	42.8	7.19
	322.0	6.73	5.59		78.2	40.25	43.0	7.20
	410.0	7.44	5.99		83.8	39.5	41.5	7.19
	512.0	8.01	6.19		86.5	39.8	41.8	7.19
	723.0	9.10	6.53		90.6	39.2	41.2	7.19
18	4.23	0.71	0.69	7.15	9.70	35.6	23.6	6.96
	16.7	1.97	1.91		26.7	35.2	23.3	6.96
	25.1	2.53	2.44		34.1	34.9	22.5	6.95
	53.2	3.50	3.31		45.3	34.2	22.6	6.96
	107.1	3.89	3.51		49.2	34.4	23.05	6.96
	137.7	3.98	3.49		48.8	45.7	24.7	6.83
	150.0	4.11	3.58		50.0	32.0	22.2	6.98
	225.0	4.66	3.86		53.0	31.6	22.2	6.99
	291.0	5.18	4.15		58.0	34.2	24.8	7.01
	352.0	5.59	4.34		61.0	34.2	22.3	6.95
	388.0	6.06	4.68		65.5	33.6	23.6	6.99
	461.0	6.54	4.90		68.6	34.4	23.5	6.98
	500.0	6.93	5.16		72.1	33.3	21.4	6.94
	729.0	8.37	5.68		79.5	34.6	22.4	6.96

TABLE I—*Continued*

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
6	4.23	0.65	0.64	6.04	10.6	51.85	28.3	6.86
	6.96	0.86	0.84		13.9	47.75	27.2	6.88
	16.65	1.70	1.65		27.2	50.7	27.2	6.85
	33.9	2.30	2.19		36.2	59.6	29.0	6.80
	69.9	2.76	2.53		41.8	53.9	26.9	6.81
	96.6	3.05	2.73		45.2	53.9	32.5	6.92
9	200.	3.62	2.91	6.20	47.0	46.3	26.2	6.89
	285.	3.98	2.97		48.0	47.7	30.3	6.94
	331.	4.30	3.13		50.5	46.6	25.9	6.88
	345.	4.60	3.38		54.5	47.3	25.2	6.86
	402.	5.02	3.60		58.0	44.5	22.7	6.83
	481.	5.32	3.61		58.2	46.6	25.8	6.87
	580.	5.59	3.54		57.1	45.5	26.9	6.91
	649.	5.94	3.64		58.7	46.1	24.5	6.86
5	4.90	0.61	0.61	6.16	9.9	120.2	39.55	6.57
	8.17	0.89	0.88		14.3	119.4	39.65	6.57
	12.74	1.18	1.16		18.8	110.8	38.25	6.60
	20.82	1.65	1.57		25.5	129.5	40.55	6.54
	36.14	2.05	1.90		30.8	123.0	40.00	6.56
	69.2	2.56	2.33		37.8	125.5	41.05	6.56
14	6.05	0.665	0.64	6.06	10.7	100.7	18.68	6.13
	13.10	1.015	0.97		16.0	100.2	18.40	6.12
	23.45	1.38	1.30		21.5	100.5	18.33	6.12
	33.7	1.63	1.51		24.9	97.2	18.10	6.13
	40.8	1.90	1.76		29.0	98.1	17.80	6.11
	48.7	2.02	1.85		30.5	97.7	17.48	6.10
	58.6	2.21	2.00		33.0	98.8	17.95	6.11
	91.0	2.44	2.12		35.1	97.4	18.00	6.13
	140.6	2.84	2.34		38.6	105.3	18.17	6.06

* Each experiment number represents measurements made on a single collection of blood.

† Sodium bicarbonate added to the blood.

In order to take account of the apparent inability of all of the hemoglobin to combine with oxygen, which characterizes the more acid samples, it is necessary to introduce a term α_0, equal to the fraction of total hemoglobin incapable of combining with oxygen. It follows that

$$\alpha_0 + \alpha_1 + \alpha_2 = 1.$$

THE OXYGEN DISSOCIATION CURVES OF TOADFISH BLOOD

The results of the experiments on the blood of the toadfish are given in Table I and the oxygen dissociation curves at constant pH are shown in Fig. 2.

These curves may be observed to have certain interesting characteristics regardless of the theory or of the equations used to describe the oxygen-hemoglobin equilibrium. The first of these is a difference in the shape of the curves at different hydrogen ion concentrations. In mammalian blood, on the contrary, curves at constant carbon dioxide tension or at constant pH are apparently of one family having the same shape.

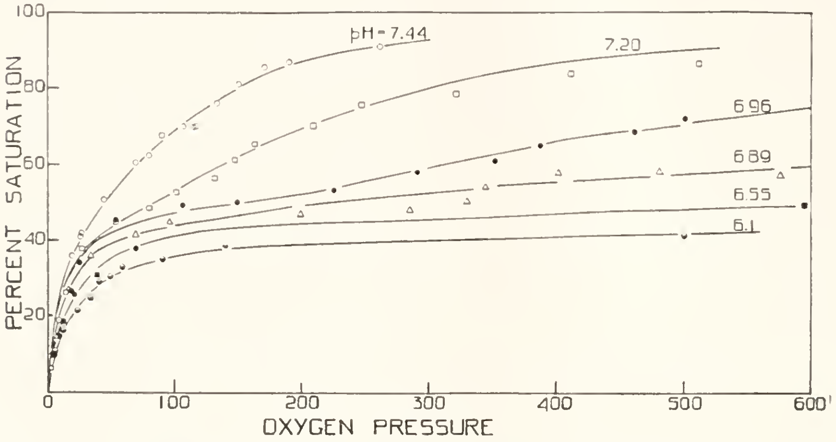

FIG. 2. Oxygen dissociation curves at constant pH of the blood of the toadfish. The curves have been drawn according to equation (3) using the following constants:

pH	α_1	α_2	α_0	K_1	$K_2 \times 10^4$	$1/K_1$	$1/K_2$
7.44	.5	.5	0	.11	.826	9	110^2
7.20	.5	.5	0	.10	.186	10	232^2
6.96	.5	.48	.02	.10	.033	10	548^2
6.89	.5	.2	.3	.071	.033	14	548^2
6.55	.5	0	.5	.0475		21	
6.1	.43	0	.57	.0475		21	

The data may be equally well described by equation (2) when the following values for the constants are used:

pH	K_1	$K_2 \times 10^2$	$K_3 \times 10^4$	$K_4 \times 10^6$
7.44	.2	1.0	.01	1.0
7.20	.1	.6	.04	.10
6.96	.1	.5	.001	.015
6.89	.1	.4	.001	.004
6.55	.1	.2	.001	.0001

A second effect of pH on these curves is a shift to the right with increasing acidity, a phenomenon well established for various mammalian bloods. On the other hand, the change in the position of the curve with change in pH is almost insignificant in the blood of the elasmobranch, *Raia ocellata* (Dill, Edwards, and Florkin, 1932). In these marine teleosts, and especially in the toadfish, the effect is very marked. As a result the curves determined at pH 7.2, 6.96, and 6.89 are not completely saturated even in the presence of 500 mm. O_2 and the shape of the curves indicates that more oxygen would be combined at higher oxygen tensions.

This is an explanation of a part of the decrease in oxygen capacity described by Root (1931). It should be noted, however, that the curves determined at pH 6.55 and 6.1 are practically flat at tensions of oxygen above 150 mm. which suggests that saturation is complete but that a part of the hemoglobin is incapable of combining with oxygen.

The next important feature of the curves is their peculiar shape. Some of them are not simple hyperbolic or sigmoid curves but are undulatory in character, having two regions concave to the abscissa. Similar types of curves have been found for the blood of some birds (Wastl and Leiner, 1931) and of certain animals containing hemocyanin (Redfield, 1933). The break between the two regions of the curves occurs at about 50 per cent saturation, and the more acid curves, in which the combined oxygen has become constant, do not rise above 50 per cent saturation. This characteristic of the curves suggests that the oxygenation of the hemoglobin consists of two steps, or that there are two components to the curves representing the behavior of different fractions of the pigment present in equal quantities. Theoretical treatment of these possibilities is provided by the equations given above.

The equation (1) developed by Hill applies only to simple curves, hyperbolic or sigmoid in shape, and thus could be used to describe only the most acid curves and these only if the decrease in oxygen capacity is assumed to be real so that higher oxygen tensions would not increase the oxygen bound.

Although Adair's theory in which the hemoglobin combines with oxygen in steps was derived for sigmoid curves, equation (2) may be used to describe the oxygen dissociation curves of the toadfish. By making K_3 and K_4 sufficiently small, the terms in which they appear become ineffective at the oxygen tensions used and the curve becomes flat at one half saturation, as in the experiment at pH 6.55. According to this treatment the decrease in oxygen capacity is only apparent and the effect really one on the magnitude of the constants and thus a "Bohr" effect. However, the curve at pH 6.1 is indubitably flat over a long range at less than 50 per cent saturation, that is, at 43 per cent, and it is also apparent from Fig. 1 that a further increase in acidity would further decrease the oxygen bound. Such curves can only be derived from the Adair equation by assuming a true loss in oxygen capacity, thus necessitating the introduction of a new constant to describe this loss.

The curves may be described equally well in terms of Redfield's equation which employs fewer constants. The smooth curves in Fig. 2 are drawn according to equation (3). When the curves have not flattened out at one atmosphere of oxygen, the fraction of hemoglobin incapable of combining with oxygen must be calculated from the shape of the dissociation curves at lower tensions and this unfortunately gives an additional degree of freedom in analyzing some of the curves.

The difference between the values for K_1 and K_2 gives the undulatory character to the curves. In some cases the hemoglobin combining with oxygen according to the first constant is almost completely saturated before the second inflection begins. By taking α_1 and α_2 each $= 0.50$, which implies that the amount of hemoglobin acting as though it combined with one molecule of oxygen at a time exactly equals that combining with two molecules, the flat places in the curves or the points where there is a change of inflection occur at about one half saturation.

The decrease in oxygen capacity takes place first at the expense of the component behaving as though $n = 2$. The value of α_1, the amount of hemoglobin behaving according to the first term of the equation, remains constant at 0.50 throughout the pH range until the amount of hemoglobin capable of combining with oxygen has been reduced to 50 per cent of the whole. Thus, the curve at pH 6.55, in which the capacity is 50 per cent of the total oxygen-combining power, is a rectangular hyperbola. Further addition of acid reduces the capacity but the curve is still of the same shape and has the same value for the constant K_1. This implies that the effect described by Root comprises a real inactivation of the hemoglobin and is due only in part to a greatly exaggerated "Bohr" effect.

All three treatments have certain common implications. The description of the curves in accordance with either equation (1), (2), or (3) requires the assumption that a portion of the hemoglobin has lost its ability to combine with oxygen in the case of the most acid solution. In the use of equation (3) it is implied that the prosthetic groups responsible for the two components of the curves are divided in equal amounts. This condition could be met if there were two forms of hemoglobin present, but the probability of two independent substances existing in such exact proportion is rather small. It is much simpler to postulate the existence of a number of oxygen-combining groups on one molecule, one half of which behave in one way and one half in the other. Since one half of the group behave as though they combined with oxygen in pairs, at least four prosthetic groups must be attributed to the molecule. In this regard the implications regarding the structure of the hemoglobin molecule are the same as those postulated by Adair in deriving equation (2).

THE OXYGEN DISSOCIATION CURVES OF THE BLOOD OF THE TAUTOG AND OF THE GOOSEFISH

The blood of the tautog and of the goosefish may be considered together since they exhibit similar characteristics. The data are presented in Tables II and III.

The most important characteristic of these curves is a change in the shape of the curve with a change in pH. Equation (1) may be transformed to the logarithmic form:

$$\text{Log}\,\frac{\text{HbO}_2}{\text{Hb}} = \log K + n \log \text{pO}_2.$$

When $\log \text{HbO}_2/\text{Hb}$ is plotted against $\log \text{pO}_2$, the resulting curve is a straight line, the slope of which is equal to n. The data for these two fish bloods have been calculated in this manner and the results plotted in Fig. 3. In both cases the most alkaline curves are straight lines and the slope is 2.0, that is, Hill's equation is applicable and n is 2.0. In both cases, also, the most acid curves are straight lines but here n is 1.0 and consequently the oxygen dissociation curves are rectangular hyperbolæ.

In both these bloods the oxygen-combining power is decreased in the more acid solutions. In the tautog the curve has changed to a rectangular hyperbola ($n = 1.0$) at pH 7.2. At this reaction the oxygen capacity is not appreciably lowered, so that the change in shape is independent of this phenomenon. In the goosefish the decrease in oxygen capacity begins to appear before the curves have assumed a shape characterized by $n = 1$, and in applying Hill's equation it is necessary to make allowances for the decreased oxygen capacity of the blood, as was done in the case of toadfish blood (p. 391). At pH 6.84, 6.80, and 6.1 the oxygen capacities are assumed to be 88, 80, and 54 per cent respectively, of those obtaining in alkaline solutions.

Table II

Oxygen Dissociation of Goosefish Blood Equilibrated at 25° C.

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
3	2.77	2.80	2.79	8.40	33.2	2.53	24.1	8.20
	7.08	5.70	5.68		67.5	3.16	25.2	8.11
	10.4	7.02	6.98		83.0	2.14	22.9	8.23
	15.94	7.90	7.84		93.3	2.41	23.2	8.20
	66.9	8.74	8.50		101.0	2.76	24.2	8.15
4 †	5.26	5.07	5.05	8.59	58.8	6.36	51.3	8.12
	6.79	5.64	5.62		65.5	8.54	56.2	8.03
	7.74	6.42	6.39		74.4	6.17	50.4	8.13
	7.89	6.35	6.32		73.6	7.23	54.3	8.09
	12.33	7.50	7.46		86.8	7.20	53.9	8.09
	14.58	7.96	7.91		92.2	6.29	52.0	8.15
	25.1	8.32	8.23		95.8	6.22	51.2	8.13
3	3.19	0.93	0.93	8.40	11.1	0.98	2.79	7.65
	21.3	5.12	5.04		60.0	0.91	2.8	7.52
	25.8	5.82	5.73		68.2	0.93	2.07	7.55
	32.3	6.36	6.25		74.3	0.61	1.6	7.62
	58.0	7.54	7.33		87.4	1.10	2.3	7.52
4 †	9.86	1.22	1.18	8.59	13.7	48.3	39.5	7.08
	25.7	3.09	3.00		35.0	45.6	37.5	7.08
	31.35	3.67	3.56		41.4	47.2	37.3	7.06
	39.85	4.45	4.31		50.3	43.5	35.0	7.07
	68.7	5.92	5.68		66.2	42.3	33.0	7.07
	109.6	7.36	6.97		81.0	36.4	30.1	7.08
	151.	8.11	7.58		88.3	33.2	28.4	7.10
	182.	8.65	8.00		93.3	33.8	28.3	7.09
4 †	12.0	1.41	1.37	8.59	15.9	49.6	39.6	7.06
	25.1	2.76	2.67		31.1	49.7	39.2	7.06
	37.4	3.45	3.32		38.6	50.7	37.8	7.03
	54.4	4.37	4.18		48.7	49.6	37.7	7.04
	104.6	7.00	6.63		77.3	42.4	32.8	7.05
	149.5	7.22	6.69		77.9	41.3	32.6	7.06
	148.2	7.43	6.90		80.3	41.1	31.5	7.04
	228.	8.38	7.57		88.3	41.2	30.3	7.03
3	4.05	0.55	0.54	8.40	6.45	50.7	26.0	6.84
	19.8	1.52	1.45		17.3	50.0	25.7	6.84
	27.35	2.15	2.05		24.4	50.7	26.2	6.84
	59.9	3.57	3.36		40.0	50.8	24.9	6.81
	102.6	4.31	3.95		47.2	50.3	24.9	6.82
	197.5	5.99	5.29		62.8	49.6	23.3	6.80
	303.5	6.92	5.84	8.28	70.5	46.7	22.0	6.79
	593.	8.73	6.62	8.19	80.7	46.4	20.7	6.76
	732.	9.05	6.45	7.94	81.3	48.5	21.3	6.75

TABLE II—*Continued*

Experiment *	O_2 Pressure	O_2 Content	O_2 Combined	O_2 Capacity	O_2 Saturation	CO_2 Pressure	CO_2 Content	pH
	mm. Hg.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*mm. Hg.*	*vol. per cent*	
4	7.0	0.72	0.70	8.59	8.15	41.0	19.8	6.80
	12.8	1.12	1.07		12.5	41.8	20.2	6.80
	34.5	2.22	2.10		24.5	39.4	19.5	6.82
	80.4	3.87	3.58		41.7	38.8	16.6	6.74
	106.5	4.39	4.02		46.8	37.6	18.1	6.80
	150.	5.01	4.48		52.1	34.9	16.6	6.78
	250.	6.34	5.45		63.5	31.2	14.1	6.77
	743.	9.08	6.44		75.0	25.1	11.6	6.78
3	34.6	1.88	1.76	8.40	20.9	390.	70.6	6.13
	64.3	2.24	2.00	8.32	23.8	374.	67.2	6.12
	106.	2.88	2.50	8.03	31.2	404.	72.0	6.11
	150.	3.39	2.86	7.96	35.8	374.	66.6	6.11
	378.	4.35	3.01	7.24	41.6	377.	68.7	6.13

* Each experiment number represents measurements made on a single collection of blood.

† Sodium bicarbonate added to the blood.

The curves at intermediate pH values are such that *n* lies between 1 and 2. The curve for the tautog at pH 7.4 is apparently a straight line of slope 1.3 so that equation (1) is applicable whereas the intermediate goosefish curves would seem to be slightly curved, indicating that a more complicated equation would give a more satisfactory description.

There is no question but that equation (2) could be used to describe these curves but their simple character renders the application of such a complicated expression of little interest.

Equation (3) may also be successfully applied here. The smooth curves in Figs. 4 and 5 are drawn according to this equation using the constants given in the legends.

Thus the dissociation curves of the tautog and the goosefish are so simple in character that they may be described by equations based on any one of the theories of the mode of combination between oxygen and hemoglobin, and in themselves give no basis for a preference of one theory over another.

These curves leave little doubt that the decrease in oxygen in acid solution is really due to a loss in ability to combine with oxygen, and not to a "Bohr effect," for certain of the curves have the same dissociation constant and differ only by the total amount of oxygen with which they are able to combine when the acidity is changed. The

TABLE III

Oxygen Dissociation of Tautog Blood Equilibrated at 25° C.

Experiment *	O_2 Pressure *mm. Hg.*	O_2 Content *vol. per cent*	O_2 Combined *vol. per cent*	O_2 Capacity *vol. per cent*	O_2 Saturation *per cent*	CO_2 Pressure *mm. Hg.*	CO_2 Content *vol. per cent*	pH
6 †	9.56	1.45	1.42	9.66	14.7	12.60	56.1	7.86
	13.13	3.04	2.99		30.9	12.5	55.0	7.85
	14.23	3.07	3.02		31.3	11.80	55.1	7.88
	21.8	4.90	4.82		50.0	10.97	52.4	7.89
	38.7	7.07	6.93		71.7	10.02	50.2	7.91
	42.9	7.73	7.58		78.5	10.35	50.1	7.90
	84.2	9.09	8.79		90.7	9.59	49.1	7.92
2	12.9	1.17	1.12	8.74	12.7	9.13	15.05	7.41
	15.2	1.52	1.47		16.7	7.62	13.50	7.45
	22.6	1.97	1.89		21.5	7.89	14.35	7.46
	46.7	4.24	4.06		46.1	8.08	12.33	7.38
	70.1	5.10	4.85		55.0	7.98	12.12	7.38
	155.	7.33	6.80		77.2	5.73	9.50	7.41
	276.	8.92	7.95		90.2	5.11	8.06	7.40
4	16.4	1.38	1.32	9.39	14.2	25.8	26.1	7.18
	52.5	2.89	2.71		29.1	26.3	26.3	7.17
	109.5	4.89	4.50		47.9	19.86	22.3	7.23
	152.5	5.62	5.08		54.1	21.1	22.2	7.20
	214.5	6.45	5.69		61.1	18.8	20.8	7.22
	268.	7.08	6.13		65.9	19.3	20.5	7.20
	381.	8.37	7.02		75.5	16.8	17.8	7.20
3	13.9	1.10	1.05	7.66	13.7	118.8	36.8	6.54
	30.5	1.78	1.67		21.8	116.6	36.2	6.54
	53.8	2.30	2.11		27.5	118.5	36.5	6.54
	87.9	2.59	2.28		29.7	137.4	38.9	6.48
	124.7	3.14	2.70		35.3	130.2	36.6	6.48
	147.0	3.46	2.94		38.4	106.8	33.6	6.55
	150.8	3.36	2.83		37.0	130.7	36.8	6.48
	163.8	3.61	3.03		39.5	117.6	36.5	6.54
	309.0	4.62	3.52		45.8	123.0	36.4	6.51
	625.0	5.91	3.69		48.0	134.2	37.6	6.46
5 ‡	23.3	0.82	0.74	9.54	7.74	107.3	14.33	5.73
	39.6	1.34	1.20	8.38	14.3	104.5	15.10	5.87
	54.2	1.39	1.20	7.87	15.2	106.0	13.60	5.67
	81.9	1.55	1.26	7.77	16.2	110.8	14.49	5.71
	101.8	2.25	1.89	8.44	22.4	107.0	14.94	5.82
	155.0	2.33	1.78	8.00	22.31	109.8	14.18	5.69
	670.0	5.06	2.68	8.52	31.4	108.2	14.46	5.76

* Each experiment number represents measurements made on a single collection of blood.

† Sodium bicarbonate added to the blood.

‡ Lactic acid added to the blood.

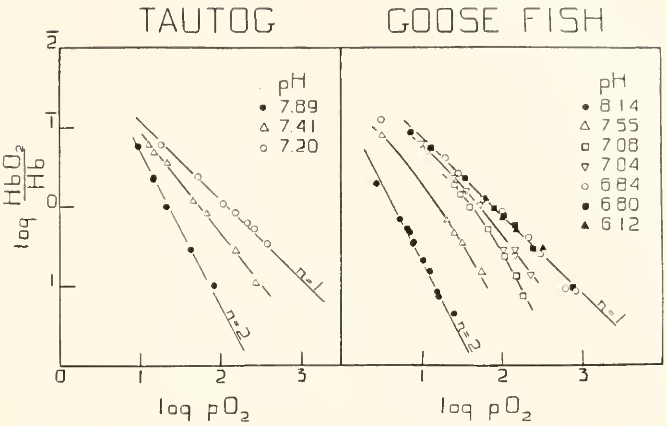

Fig. 3. For explanation of this figure, see text.

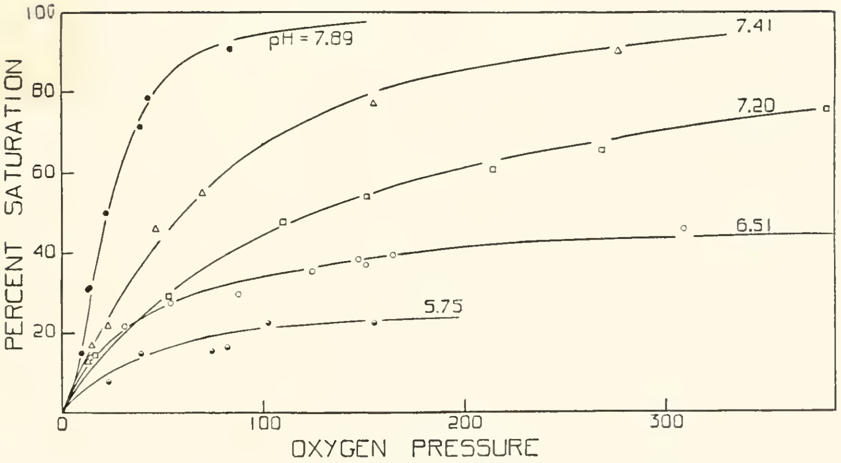

Fig. 4. Oxygen dissociation curves at constant pH of the blood of the tautog. The curves have been drawn according to equation (3) using the following constants:

pH	α_1	α_2	α_0	K_1	$K_2 \times 10^2$	$1/K_1$	$1/K_2$
7.89	0	1.0	0		.19		23^2
7.41	.5	.5	0	.0286	.0156	35	80^2
7.20	1.0	0	0	.008		125	
6.51	.5	0	.5	.022		45	
5.75	.3	0	.7	.022		45	

more acid curves for tautog blood are rectangular hyperbolæ and K_1 is the same at pH 6.5 and 6.1 although the oxygen capacity is diminished. The same is true of the goosefish blood at pH 6.84, 6.80, and 6.12.

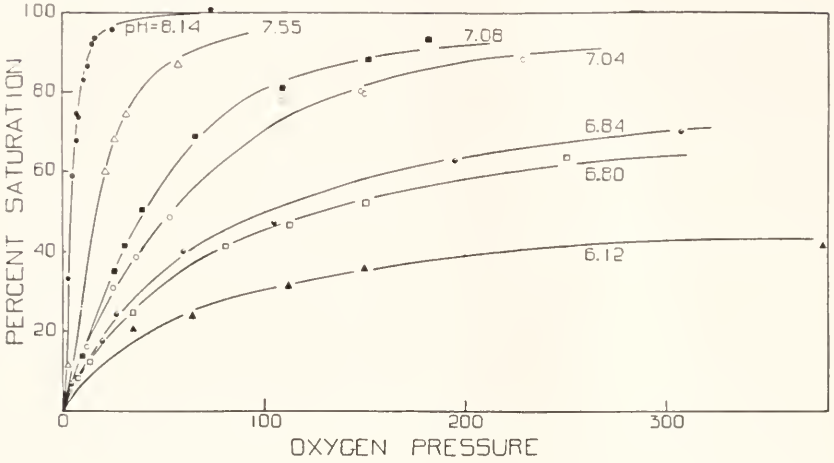

FIG. 5. Oxygen dissociation curves of the blood of the goosefish at constant pH. The curves have been drawn according to equation (3) using the following constants:

pH	α_1	α_2	α_0	K_1	K_2	$1/K_1$	$1/K_2$
8.14	0	1.0	0		.05		4.47^2
7.55	.25	.75	0	.067	.0011	15	30^2
7.07	.45	.55	0	.04	.0004	25	50^2
7.04	.50	.50	0	.04	.000158	25	80^2
6.84	.88	0	.12	.0131		76	
6.80	.80	0	.20	.0131		76	
6.12	.54	0	.46	.0131		76	

The change in the position of the curves with pH is very marked in these bloods as well as in that of the toadfish. In the tautog there is an additional phenomenon. The oxygen dissociation constant for the curve at pH 7.2 is considerably smaller than that for the curves at lower pH values. That is, at first the curves are shifted to the right with increasing acidity but at higher acid concentrations there is a reversal of the effect. A similar reversal of the Bohr effect was found by Rona and Ylppö (1916) working on dog blood, by Ferry and Green (1929) for horse hemoglobin, and by Stedman and Stedman (1926) and Hogben (1926) for crustacean hemocyanin.

The most interesting single characteristic of these curves is the change in shape from a rectangular hyperbola to a sigmoid curve. Unlike the toadfish, in which one component behaves as though $n = 2$ and one component as though $n = 1$ at all pH values, the change in shape must be due to a change in behavior of the component groups.

Thus, whatever the theory used as a basis for the description of the curves, it may be observed that acidity has a threefold effect on the blood; first, a change in the value of the oxygen dissociation constant, second, a decrease in oxygen capacity, third, a change in the shape of the curves describing the equilibrium with oxygen.

Theoretical Deductions Concerning the Loss in Oxygen Capacity in Acid Solutions

Henderson (1920) has shown that the effect of acid on the combining power of hemoglobin with oxygen could be described in terms of four mass law expressions; one for the combining power of oxygen with acid hemoglobin, HHb, one for oxygen with salt hemoglobin, BHb, and one each for the acid dissociation of oxygenated and reduced hemoglobin, $HHbO_2$ and HHb respectively. Thus the ease with which oxygen combines with hemoglobin depends upon the dissociation of the latter as an acid and oxyhemoglobin is a stronger acid than reduced hemoglobin.

The situation becomes rather complicated when more than one molecule of oxygen is considered to combine with one molecule of hemoglobin. By making certain assumptions concerning the interdependence of the four prosthetic groups, it is possible to derive an expression predicting the relation between oxygen capacity and hydrogen ion concentration exhibited by the data in Fig. 1. By making a few assumptions it is possible to explain this phenomenon of reversible loss of oxygen capacity in relatively simple chemical terms. The fundamental conception is that the oxygen combination is intimately dependent upon the acid dissociation of the hemoglobin as in mammalian blood, but that the effect is so exaggerated that oxygen, at ordinary oxygen tensions, will not combine with the hemoglobin unless the latter is in the ionized state. This is, of course, not the only possible theory but it is set forth here as a simple interpretation of the mechanism of a phenomenon apparently very different from our usual conception of the equilibrium between oxygen and hemoglobin.

Let it be assumed:

1. That each hemoglobin molecule contains four groups capable of

combining with oxygen and that each of these groups is associated with the dissociation of two hydrogen ions.[2]

2. Assume further that only the ionized prosthetic groups, that is, the groups from which hydrogen ions have been dissociated, can combine with oxygen.[3] Then in the presence of oxygen sufficient to combine with all the ionized hemoglobin the number of forms present is reduced to H_8Hb, $H_6HbO_2^{=}$, $H_4Hb(O_2)_2^{\equiv}$, $H_2Hb(O_2)_3^{\equiv=}$, and $Hb(O_2)_4^{\equiv\equiv}$.

3. Assume finally that the first two pairs of H ions dissociate from the hemoglobin separately but the last two pairs dissociate in a single step; then there never is present an appreciable amount of the form which can combine with three molecules of oxygen. This is in accordance with the usage of equation (3) as applied to the oxygen dissociation curves of toadfish blood.

The equilibrium reactions for the acid and oxygen dissociation of the hemoglobin molecule according to these restrictions are reduced to

$$H_8Hb = H_6Hb^{=} + 2H^+ \qquad H_6Hb^{=} + O_2 = H_6Hb(O_2)^{=}$$

$$H_6Hb(O_2)^{=} = H_4HbO_2^{==} + 2H^+ \qquad H_4HbO_2^{==} + O_2 = H_4Hb(O_2)_2^{==}$$

$$H_4Hb(O_2)_2^{==} = Hb(O_2)_2^{\equiv\equiv} + 4H^+ \qquad Hb(O_2)_2^{\equiv\equiv} + 2O_2 = Hb(O_2)_4^{\equiv\equiv}$$

If the oxygen tension is sufficiently high to convert practically all of the hemoglobin from which the hydrogen has been dissociated into the corresponding oxygenated form, the intermediate product of each pair of equations is never present in appreciable amount and the following equilibrium reactions and mass law expressions may be assumed to describe the limiting conditions:

$$H_8Hb = H_6HbO_2^{=} + 2H^+ \qquad \frac{[H_6HbO_2^{=}]}{[H_8Hb]} = \frac{K_1}{[H^+]^2}$$

$$H_6HbO_2^{=} = H_4Hb(O_2)_2^{=} + 2H^+ \qquad \frac{[H_4Hb(O_2)_2^{==}]}{[H_6HbO_2^{=}]} = \frac{K_2}{[H^+]^2}$$

$$H_4Hb(O_2)_2^{\equiv} = Hb(O_2)_4^{\equiv\equiv} + 4H^+ \qquad \frac{[Hb(O_2)_4^{\equiv\equiv}]}{[H_4Hb(O_2)_2^{==}]} = \frac{K_3K_4}{[H^+]^4}$$

---

[2] The assumption that the combination of each oxygen molecule is accompanied by the dissociation of two hydrogen ions is in accordance with the finding of Ferry and co-workers (1929) that K_1 in equation (2), as applied to horse hemoglobin and to sheep blood, varies with the square of the hydrogen ion concentration, and with the observation of Redfield and Ingalls (1932) that the oxygen dissociation constant for certain hemocyanins also varies with the square of the hydrogen ion concentration.

[3] This also has a parallel in the hemocyanins, where the addition of acid produces a colorless compound incapable of combining with oxygen. See the end of this discussion.

The relative concentrations of the different forms present at any given pH may be readily calculated either by the logarithmic form usually employed in treating titration curves or in an algebraic form similar to the oxygen dissociation equations. Thus for the first equilibrium if

$$[H_8Hb] + [H_6HbO_2^{=}] = 1,$$

then

$$\frac{[H_6HbO_2^{=}]}{1 - [H_6HbO_2^{=}]} = \frac{K_1}{[H^+]^2} \text{ and } [H_6HbO_2^{=}] = \frac{K_1}{[H^+]^2 + K_1}$$

and similarly for the other expressions,

$$[H_4Hb(O_2)_2^{\equiv}] = \frac{K_2}{[H^+]^2 + K_2}, \text{ and } [Hb(O_2)_4^{\equiv\equiv}] = \frac{K_3K_4}{[H^+]^4 + K_3K_4}.$$

The total amount of oxygen bound at any given pH is the sum of all of the oxygenated forms.

$$H_6HbO_2^{=} + H_4Hb(O_2)_2^{\equiv} + Hb(O_2)_4^{\equiv\equiv}$$

Since only one quarter of the total oxygen bound can be in the form $H_6HbO_2^{=}$, the concentration as derived from the equilibrium expression must be multiplied by 0.25 as must also the concentration of $H_4Hb(O_2)_2^{\equiv}$, while the concentration of $Hb(O_2)_4^{\equiv\equiv}$ must be multiplied by 0.50. Substituting these values and the mass law expressions for the various oxygenated forms the total oxygen combined, Z, becomes

$$Z = .25\frac{K_1}{[H^+]^2 + K_1} + .25\frac{K_2}{[H^+]^2 + K_2} + .50\frac{K_3K_4}{[H^+]^4 + K_3K_4}.$$

In applying these equations to the data in Fig. 1, it must be kept in mind that the shape of the oxygen dissociation curves indicates that the hemoglobin would combine with slightly more oxygen if the oxygen tension were sufficiently increased. The theoretical oxygen capacity may be calculated in the more acid range because the curves are simple rectangular hyperbolæ and in all three bloods the oxygen dissociation constant has the same value in the most acid curves. If it be assumed that the constant is the same at intervening and lower pH values, knowing the oxygen tension and the concentration of oxygenated hemoglobin, it is possible to calculate the limiting amount of oxygen with which the hemoglobin can combine at these reactions. These values have been calculated using the data given in Fig. 1 for the goosefish below pH 6.8 where $K_1 = .013$, for the tautog below pH 6.55 where $K_1 = .022$, and for the toadfish below 6.5 where

$K_1 = .047$. At higher pH values the capacities have been calculated somewhat less accurately. The points between pH 6.55 and 6.96 for the tautog have been calculated using the K_1 values at both these reactions, then averaging the results which are then ± 2 per cent of the correct value. At oxygen tensions of 700 mm. the first half of the toadfish hemoglobin is practically saturated and the capacity between pH 6.55 and 7.0 has been calculated according to equation (3) using K_2 as 3.33×10^{-6}. All of the calculated points are represented in Fig. 6.

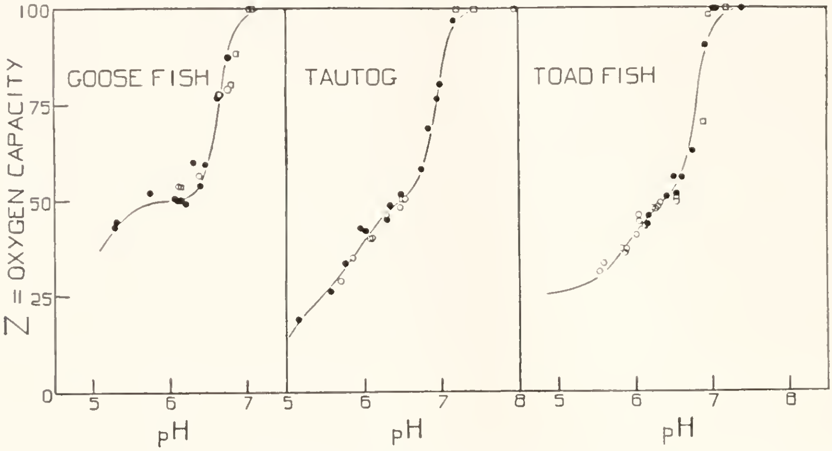

FIG. 6. The oxygen capacity of certain fish bloods. The points are calculated as described in the text:

- ● from the blood equilibrated with oxygen as given in Fig. 1.
- ○ from the blood equilibrated with air as given in Fig. 1.
- □ from the oxygen dissociation curves in Figs. 2, 4, and 5.

The curves are drawn according to equation (4).

The curves in this figure are drawn according to equation (4) where $\sqrt{K_2}$ and $\sqrt[4]{K_3K_4}$ are for the goosefish $10^{-5.1}$ and $10^{-6.65}$ respectively; for the tautog, $10^{-5.93}$ and $10^{-6.95}$ respectively, and for the toadfish $10^{-5.85}$ and $10^{-6.8}$ respectively. $\sqrt{K_1}$ is approximately 10^{-5} for the tautog but is too large to be determined for the other two fishes.

The applicability of this type of equation to the oxygen capacity supports the interpretation of the oxygen dissociation curve as developed in the case of the toadfish. The equation is also adequate to describe the relation observed in tautog and goosefish blood, although the assumptions used in deriving curves to describe the oxygen equilibrium are somewhat different from those employed for toadfish blood. This difference suggests that in some cases there may be an interdependence of the acid dissociation of the prosthetic groups which is not paralleled by an interdependence in the combination of oxygen.

The most important implication of the curves in Fig. 6 is the same as that derived from the undulatory character of the oxygen dissociation curves of the toadfish, namely, hemoglobin contains prosthetic groups differing in their behavior with respect to hydrogen dissociation and oxygen combination.

The evidence for this is the inflection of the tautog and toadfish curves at 50 per cent saturation and the actual break in the goosefish curve at the same point. Thus the effect of the addition of acid, as originally suggested (Root, 1931), may be interpreted as the inactivation of prosthetic groups on the hemoglobin molecule. This inactivation is probably due to the undissociated character of the hemoglobin as an acid, oxygen combining with only the ionized form of hemoglobin.

A similar inactivation of respiratory pigment in the presence of acid has been described by Redfield, Mason, and Ingalls (1932). The hemocyanin of *Limulus polyphemus* reacts with hydrochloric acid to form a component that is colorless and does not react with dissociable oxygen. The colorless component may be separated from a partially acidified hemocyanin solution by the addition of a strong solution of sodium chloride. When such a separation has been effected, analysis of the nitrogen in the filtrate indicates that its protein content has been diminished to just such an extent as the hemocyanin has been decolorized. Here, also, the combination of each oxygen molecule appears to be dependent upon the dissociation of two hydrogens, for the oxygen dissociation curves are rectangular hyperbolæ and the equilibrium between hydrochloric acid, hemocyanin, and the resulting colorless component may be described by an equation in which the hemocyanin is behaving as a divalent acid or base.

We wish to express our thanks to Professor Alfred C. Redfield for his generous interest and direction throughout the course of this investigation.

SUMMARY

1. The oxygen dissociation curves of the blood of certain marine teleosts, the toadfish, the goosefish, and the tautog, have been studied.

2. The oxygen dissociation curves for the toadfish are undulatory in character with two areas concave to the abscissa. The second inflection begins at approximately one half saturation.

3. The oxygen dissociation curves for the goosefish and the tautog change shape with change in pH. At alkaline reactions the curves are sigmoid whereas in acid solutions they are rectangular hyperbolæ.

4. The form of the oxygen dissociation curves at constant pH can be described by Hill's equation only under a limited number of circumstances. The curves for the toadfish may be described in terms of equations derived from the assumption that each molecule of hemoglobin combines with four molecules of oxygen.

5. The position of the dissociation curves of all of these fish bloods is markedly affected by pH, and, in addition, with increasing acidity the oxygen capacity is reduced.

6. The manner in which the addition of acid lowers the oxygen capacity may be deduced from the assumption that oxygenation is dependent upon the dissociation of hemoglobin as an acid.

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OBSERVATIONS UPON STENOSTOMUM ŒSOPHAGIUM

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The genus *Stenostomum* has been studied frequently since its first species was described by Ant. Dugès in 1828 as *Derostoma leucops*. There is considerable confusion in the records concerning the anatomy and life-history of the species of this genus. Our observations have to do with certain anatomical details and phenomena dealing with sexual propagation.

Material and Methods

The species *S. œsophagium* was described by Kepner and Carter in 1930, their description being based upon the first of the two clones employed by us. This first clone arose from an individual collected in September, 1929, and was maintained, in numerous lines, until July, 1930, when it died during the intense heat of the season. The second clone was established from a collection made in the last week of August, 1930. At the present writing, December, 1932, this clone is still running. Cultures have been maintained in wheat infusions,[1] little attention being paid to the hydrogen ion concentration. The range of pH for satisfactory cultures is from 5.6–7.6. Our most thrifty specimens have appeared in culture media that were on the acid side as low as pH 6.3.

The best material for sectioning was obtained by using hot (about 50° C.) Zenker's fluid. Iron hæmatoxylin, with eosin as a counterstain, was employed for the most part.

General Anatomy

The spindle-shaped body of this species measures about 1.5 mm. in length when no obvious fission plane is present. A specimen that has experienced inanition is colorless. A well-fed individual, on the other hand, has a yellow-brown tint due to the presence of absorbed food within the enteric epithelium. There are three external apertures: a ventral sub-terminal mouth; a ventral nephropore near the middle of the caudal region; and a dorsal, male gonopore that lies over the mouth. The body is covered with cilia.

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[1] Four grains of wheat were boiled in 100 cc. of spring water and inoculated with bacteria, rotifers, and protozoa.

The epidermis bears numerous rhabdites (Fig. 1*B*, *r*) which lie in slender, parallel groups at the bases of the epidermal cells and expand distally to become almost uniformly distributed beneath the outer surface of the epidermis. In the fixed condition, the epidermis, from which the rhabdites have been discharged, shows deeply-staining chromatic lines (Fig. 2, *l*) that converge in each cell to form a common stem lying by the nucleus. However, these common stems show no morphological connection with the nuclei.

The alimentary canal consists of a mouth, pharynx, œsophagus, and enteron. These are all lined with a ciliated epithelium. The wall of the pharynx (Figs. 1 and 3, *ph*), is more muscular than that of the œsophagus. Furthermore, many unicellular glands (Fig. 3, *pg*) are loosely applied to the outer surface of the pharynx. These glands open into the lumen of the pharynx at its anterior end directly behind a small, dorsally directed diverticulum (Fig. 3, *d*). None of these glands crowd back over the œsophagus (Figs. 1 and 3, *œ*). Food-objects are delivered by the pharynx to the œsophagus, where they are held for a time before they are passed on to the enteron. A sphincter guards the passageway between these two regions of the alimentary tract. The enteron is an oval sac with a lining of ciliated endodermal cells. This epithelium (Figs. 1 and 3, *en*) in the living animal, under the usual vegetative conditions, presents a densely granular appearance. It is made up of tall, ciliated columnar cells (Fig. 4, *enc*), which may leave the epithelium and pass out into the pseudocœle. Figure 4 shows a cell

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Explanation of Figures, Plate I

Fig. 1. *A*. Dorsal aspect of a living specimen. *en*, enteron; *mgs*, male genital system; *oe*, œsophagus; *ov*, *ov′*, *ov″*, ovaries; *ph*, pharynx; *pn*, protonephridium. × 80. After Kepner and Carter (1930).

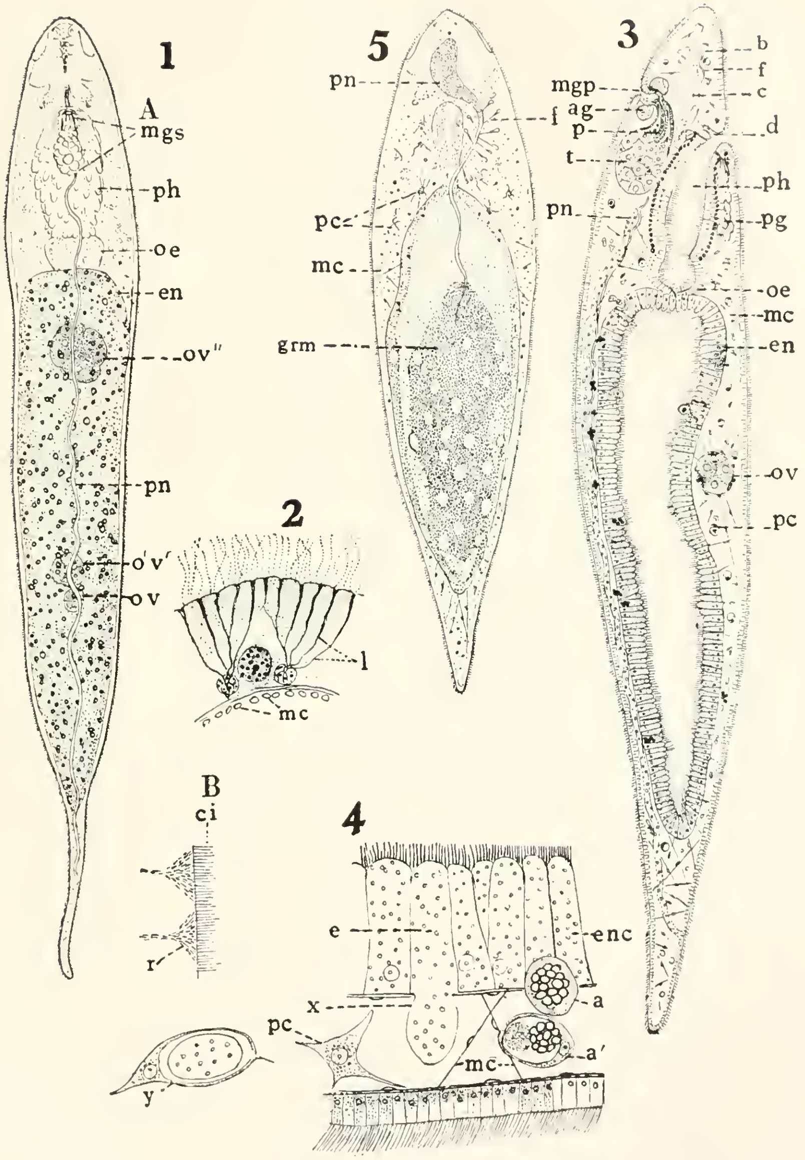
B. Region of living epidermis showing cilia (*ci*), and rhabdites (*r*).

Fig. 3. Sagittal section involving median plane. *ag*, accessory gland of male genital atrium; *b*, flexed part of protonephridium; *c*, neural commissure; *d*, dorsal diverticulum of pharynx; *f*, capillary region of protonephridium; *en*, enteron; *mgp*, male genital pore; *oe*, œsophagus; *ov*, ovary; *p*, penis; *pc*, phagocyte; *pg*, glands of pharynx; *ph*, pharynx; *pn*, main stem of protonephridium; *t*, testis. × 80.

Fig. 4. A region involving epidermis, pseudocœle, and endoderm of a living specimen as seen under water immersion objective. *a*, a wandering cell leaving epithelium of enteron, loaded with small bodies that it had taken up within the endoderm; *a′*, a similar cell that lies within the pseudocœle; *e*, endodermal cell leaving the epithelium, later its projecting end was sheared off; *enc*, endermal cell; *mc*, muscle cells; *pc*, phagocyte, which at *y* had ingested the fragment of endodermal cell that had been broken from *x*.

Fig. 5. Dorsal aspect of a specimen that had but recently deposited an egg. *f*, flame-cell; *grm*, granular material within the very thin-walled enteron; *mc*, muscle cells; *pc*, phagocytes; *pn*, region of protonephridium distended by granular inclusions. × 96.

PLATE I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that was leaving the epithelium while being observed under a water immersion objective. The shearing movements of the body-wall, however, tore the projecting part from the cell. This fragment of endodermal cell was soon ingested by the phagocyte (*pc*), which lay near, with the result shown in Fig. 4 at *y*. Between the ciliated cells of the enteron's epithelium other small cells may be found. We have seen these small cells appropriate refractive bodies, as they lay within the enteric epithelium, and then migrate from the wall of the enteron into the pseudocœle as shown in Fig. 4 at *a* and *a′*. This is taken up in greater detail by J. S. Carter (1933).

There are two bi-lobed cephalic ganglia, lying anterior to the mouth, with their larger anterior lobes in contact with the ciliated pits. An " eye-spot " is attached to each posterior lobe. The cephalic ganglia are connected by a transverse commissure (Fig. 3, *c*).

The protonephridium (Figs. 1 and 3, *pn*) is a conspicuous organ of the pseudocœle. This protonephridium consists of two regions, (1) a main stem and (2) a capillary-like portion. The main stem is the more conspicuous because of both its relative size and density. This entire organ lies in the mid-line, dorsal to the organs of the pseudocœle, except where it passes beneath the neural commissure. Anterior to this commissure, the protonephridium is bent upon itself. This deflection (Fig. 3, *b*) marks the boundary between the two regions of the protonephridium. The main stem has a thick cytoplasmic wall within which lie many nuclei, but in which no cell boundaries have been seen either in fixed or living material. The deflected part of the protonephridium appears to be a thin-walled syncytium. This capillary-like, posteriorly deflected region lies ventral to the main stem and is closely applied to it at irregular intervals. It receives scattered branches that have a similar structure. It is into this capillary-like portion of the protonephridium that the flame-cells (Figs. 3 and 5, *f*) empty. The anatomy of the

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Explanation of Figures, Plate II

Fig. 6. A phagocyte lying over a peripheral muscle cell (*m*). × 440.

Fig. 7. A median sagittal section of male reproductive system. *ag*, accessory gland; *mgp*, male gonopore; *p*, penis; *sp*, spermatids; *sv*, seminal vesicle; *t*, testis. × 416.

Fig. 8. Ventral aspect of a specimen that had but recently deposited an egg. *m*, mouth; *wo*, mesenchymal structure left in region in which egg had lain. × 80.

Fig. 9. A copulating pair in which the larger male is anchored to the anterior right side of the smaller one by the former's slender, colorless penis.

Fig. 10. Lateral aspect of mature female. Note reduced anterior or cephalic region. *e*, egg emerging as peristaltic waves travel towards it (indicated by arrows).

Fig. 11. A phagocyte that has ingested a muscle cell. *m*, muscle cell; *n*, nucleus of phagocyte. × 440.

PLATE II

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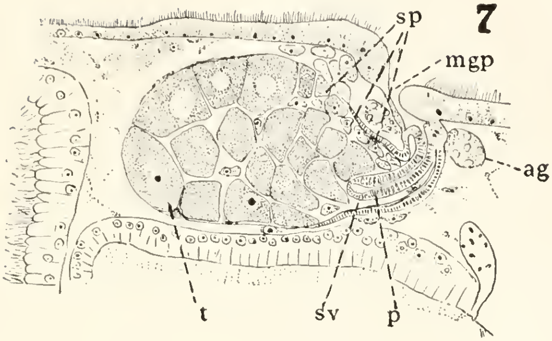
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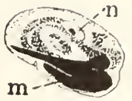

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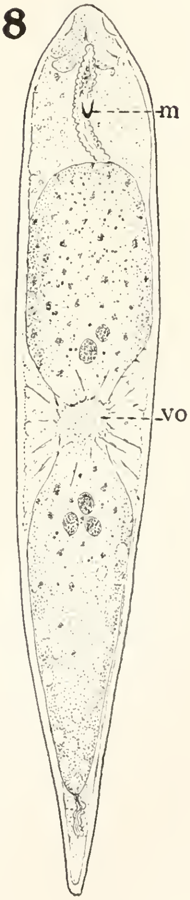

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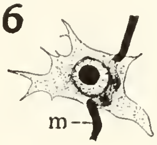

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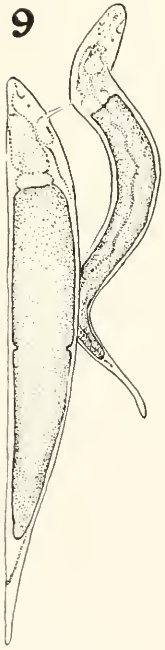

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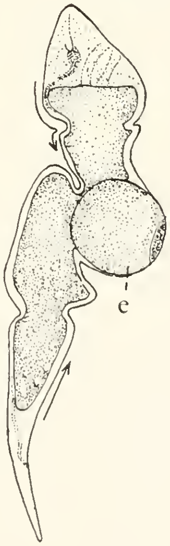

protonephridium of this *Stenostomum* is, therefore, similar to that described for *S. leucops* by Westblad (1923). Granular material has been observed frequently within the ciliated lumen of the thick-walled region but never within the lumen of the thin-walled region. This observation supports Reisinger's (1922, 1923) statement that the thick-walled region has an excretory function, while the thin-walled region has a filtering function.

Slender, non-striated, muscle-cells are associated with the body-wall, the walls of the enteron, œsophagus, and pharynx; while others (Figs. 3 and 5, *mc*) radiate from the alimentary canal to the body-wall.

Several types of leucocytes (Figs. 3, 4, and 5, *pc*) drift more or less passively within the pseudocœlomic fluid or crawl, amœba-like, over surfaces of muscle cells, between epithelial cells or to and into any other regions of the body. Figures 6 and 11 show two of these leucocytes. The gonads also lie within the pseudocœle.

Reproductive Organs

Vejdovsky (1880) was the first to have seen the male reproductive system, but he failed to recognize it as such and designated it the "ovale Drüse." Von Graff (1882) described only the female organs, while Sillimann (1885) saw the testis and expressed the opinion that the "ovale Drüse" of Vejdovsky was a sex organ. Voigt (1894) recognized both male and female gonads; while Keller (1894) gives a description of both the testis and the ovary. Landsberg (1887) records, "Hoden sah ich in derselben Kette, in der sich auch das Ovarium befand." Voigt (1894) likewise observed that *Stenostomum* was monœcious, while Sekera (1903) confirmed these observations. Von Graff (1913) states that "Die Geschlechtsorgane sind nur teilweise bekannt" (s. 18). Meixner (1924) records, "Hiezu sei vorläufig bemerkt, dass die dorsale Lage der männlichen Geschlechtsöffnung im Vorderkörper nur für etwa vier *Stenostomum-Arten* erwiesen ist" (s. 124). Sonneborn (1930) says, "Little is known concerning sexual reproduction in these organisms" (p. 59). Sabussow (1897) gave the most nearly complete description of the male reproductive system. He recognized five parts to this system in *S. leucops:* (1) a single testis; (2) a spheroidal seminal vesicle; (3) a tubular penis, lacking chitin, measuring "13 μ long and 11 μ breit"; (4) a penis-sheath; and (5) a small "antrum masculinum."

The male reproductive system of *S. œsophagium* corresponds to the description of that of *S. leucops* as given above by Sabussow (1897), except for the short tubular penis depicted and described by this author. We find that lying within the terminal duct, or neck, of the male organ is a highly muscular, tubular penis (Figs. 3 and 7, *p*). This occupies a position similar to that of the slime (*Schl* of Sabussow's Taf. 5, Fig. 1) which this author depicts and describes. We are therefore inclined to consider that Sabussow had mistaken the real penis of *S. leucops* for slime. The penis is an inverted tube capable of being everted. Its base, in the resting condition, gives the suggestion of a short tubular penis with a shallow penis-sheath. In reality it is clearly seen that in *S. œsophagium* there is a long tubular penis and that the penis-sheath shown in Sabussow's Fig. 1 is but a part of the genital antrum, the penis being housed, as a matter of fact, within the muscular seminal vesicle (Fig. 7, *sv*). In our Fig. 7 some spermatids are shown lying dorsal to the penis at *sp*.

Individuals which arise from eggs in the laboratory may become sexually mature, as males, in about three weeks. For example, an individual that emerged from the egg on December 16 was found to be sexually mature, as a male, on January 6. Sexually mature males may propagate asexually for many generations before female gonads appear.

There are no gonoducts associated with the female gonads. When oögonia are first seen in the living specimen, they lie beneath the ventral mid-line of the enteron within the pseudocœle. The growing primary oöcytes become enclosed in a loose, muscular tunic that is, in turn, supported by radiating non-striated myocytes. This rather indefinite aggregate of cells constitutes the ovary (Fig. 3, *ov*). We are not, as yet, in a position to state whether the oögonia are of mesenchymal or of endodermal origin. We are inclined to believe that they are diapeditic cells of the enteric epithelium. This subject will be taken up by one of us later.

Sekera (1903) observed that during the development of the "egg" four cells appear, one of which may function as the gamete and the remaining three may be used as yolk material. Our observations show that by the time the developing ovary becomes discernible in the living specimen, it is composed of two cells (Fig. 1, *ov*). After it has grown but a little more, the ovary contains four equal cells (Fig. 1, *ov′* and *ov″*). The gonad suggests the appearance of an embryo of four blastomeres. These cells, however, are not blastomeres. Despite the fact that there are thus always four cells in an advanced ovary, no ovary gives rise to more than one zygote. Only one of the four cells becomes the functional gamete, the other three having disappeared before egg-deposition. Both Sekera (1903) and Van Cleave (1929) indicate that at least the first cleavage of the zygote occurs before egg-deposition. We, however, are not in a position to confirm this.

A single ovary usually develops, but we have observed as many as seven ovaries develop and reach maturity in a given worm. The ovary usually lies in the anterior zoöid in specimens that present both a fission-plane and an ovary. However, specimens have been found frequently that displayed a well-defined fission-plane with an ovary in each zoöid. In these examples the anterior ovary was usually the more advanced (larger).

COPULATION

There being no female gonoducts, copulation must be peculiar. We have observed two cases of copulation.

On one occasion, while a specimen that showed no discernible ovary was feeding, a second individual in a similar sexual condition (lacking a discernible ovary, but possessing a complete male genital system) approached the mass of food in such a way that its left anterior region lay near, but not in contact with the anterior end of the first animal. The penis of the first animal was everted with a high velocity so that its end pierced or adhered to the epidermis of the second male specimen (Fig. 9). Each animal, thereupon, made efforts to free itself as the two were held together by the translucent penis of the first animal. Their efforts eventually resulted in separation. The penis was then quickly drawn back into the seminal vesicle.

On another occasion we saw a similar series of phenomena differing only in that the point of contact of the penis with the second animal's body was at a different level from that involved in our first observation.

Voigt (1894) says, "Die männlichen Geschlechtsprodukte reifen erheblich früher als die weiblichen, so dass eine Selbstbefruchtung ausgeschlossen erscheint" (s. 747). Keller (1894) also says, "Die männliche Geschlechtsreife tritt erheblich früher auf, als die weibliche. Selbstbefruchtung ist somit nicht wohl anzunehmen" (s. 398). Sekera (1906) says, however, ". . . kommt es nach der Ausbildung der Dottermasse in den Keimzellen zur Selbstbefruchtung in der Weise, dass die Hodenfollikel platzen und reife Spermatozoen in der Leibeshöhle herumschwärmen, bis sie in die eine oder andre Keimzelle der einfachen Ovarien, welche nur aus 4 Keimzellen bestehen, hineindringen und zur Ausbildung eines Eichens mit dicker Eischale beitragen" (s. 142).

Our observations of copulation support the opinion of the first two authors. Furthermore, since copulation took place between individuals, each of which had no discernible ovary, it is suggested that the presence of foreign spermatozoa may be necessary for the development of latent oögonia. This suggestion is strengthened by the fact that we have no definite examples of isolated individuals becoming sexually mature as females; but our evidence in this connection is not sufficiently extensive to be convincing.

Egg-Deposition

The absence of female gonoducts has long attracted the attention of investigators. Voigt (1894) states, " Nach der Eiablage sterben die Tiere nicht ab, sondern sie fangen schon vor Beendigung derselben an, sich wieder durch Teilung fortzupflanzen " (s. 747). Von Graff (1913), Sekera (1926), and Van Cleave (1929) record that the egg is discharged by rupturing the epidermis, resulting in the death of the parent. Carter (1930) observed that while egg-deposition brings on a critical period, it is not necessarily fatal to the mother.

We have observed the details of egg-deposition. On January 25, 1931, we discovered a specimen that had an ovary surrounded by a translucent shell. The ovary's presence caused a conspicuous protuberance near the middle of the animal. The region in the immediate vicinity of the ovary displayed great and sustained muscular contraction. The regions anterior and posterior to the ovary presented peristalsis, the waves of which traveled towards the large ovary (Fig. 10). While we took turns watching this specimen (which was quiet, except for the peristalsis), one of us saw the epidermis open and slip back over the egg and close behind it as though the epidermis were an elastic membrane with a small pore through which the egg had passed by distending the pore. The pore closed after the egg's passage had been effected, leaving no wound in the body-wall. Neither cells nor plasma could be seen to have escaped with the egg. Within the pseudocœle lay the vestige of the muscular tunic of the ovary (Fig. 8, *vo*). We have made other similar observations of egg-deposition.

The period of incubation of the eggs, under laboratory conditions, varies greatly. Van Cleave (1929) obtained two incubation periods of twenty-five days each. We have had a period as short as fifteen days and one that extended well over a month. However, few eggs deposited under laboratory conditions developed.

The Influence of the Female Gonads upon the Parent

The ovary has a marked influence, both upon the life of the clone and that of the individual, for its presence influences both the fission-rate and the anatomy of the individuals. Van Cleave (1929) records that fission is inhibited by the ovary. Landsberg (1887), on the other hand, observed chains of zoöids in which the zoöids had female gonads. We have recorded frequent cases of fission in which the ovary lay in the anterior zoöid; so we may say that when an ovary appears anterior to an incipient fission-plane, this fission-plane is not inhibited in all cases; but a second fission-plane does not appear.

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When, however, an ovary develops posterior to the fission-plane the usual events may not occur in the plane of division. The following is an example of such influence of a posterior ovary. January 24, 1931, we discovered an animal that was unusually long and had a fission-plane. Ordinarily a specimen with an advanced fission-plane measures about 3 to 4 mm. in length. This specimen, however, was 7 mm. long. The posterior zoöid had a conspicuous ovary. The anterior zoöid had a minute ovary that could be seen only with high magnification. The fission-plane's constriction indicated that it was an advanced one, but there were rudiments neither of ciliated pits, pharynx, cephalic ganglia, nor testis posterior to this plane of constriction. There was a small testes in the anterior zoöid. The enteron of the posterior zoöid housed within its wall refractive, oval bodies. On January 25 the specimen was indifferent to food. The posterior ovary had grown while the anterior one had not. On January 26 the posterior ovary had discharged its egg, the zoöid showing the depression within which the egg had been lying. The anterior ovary had not grown, but by January 27 the anterior ovary had enlarged. The posterior zoöid still showed the displacement of tissues that had been caused by the presence of the ovary, but no cephalic organs had developed. On January 28, the specimen had divided. At this time, the posterior zoöid lacked all cephalic organs. However, two weeks later this posterior zoöid had developed a pharynx, ciliated pits, and cephalic ganglia. This is but one of frequent similar examples that we have seen. Thus it appears that when an ovary is more advanced in the posterior zoöid, the formation of organs or differentiation of tissues in the region of the fission-plane is inhibited.

Sekera (1903) observed " Bald darauf beginnt auch der Pharynx zu degenerieren und das Individuum nimmt keine Nahrung auf, indem es durchsichtig und verkümmert zu werden pflegt " (s. 543).

We have found that the presence of a developing ovary makes an increased demand for material that must be supplied at the expense of the substance of the body as a whole. The leucocytes of the mesenchyme increase in number in the vicinity of the pharynx as the ovary grows. These become phagocytic and attack the radiating muscles of the pharynx and ingest them (Fig. 11). This phagocytosis eventually greatly reduces the size of the pharynx and œsophagus The testis completely disappears. The main stem or thick-walled region of the protonephridium is resorbed at irregular levels, sometimes leaving only an enlarged sack-like region (Fig. 5, *pn*) as a vestige of the main stem, and the capillary region bearing flame-cells (Fig. 5, *f*). This vestige becomes greatly distended and contains a dense mass of minutely crystalline material (Fig. 5, *pn*). With advanced phagocytosis, the wall of the enteron becomes quite thin and a mass of granular material (Fig. 5, *grm*) appears within its lumen. This material resembles that found within the distended anterior region of the main stem of the protonephridium at this time. It may be that with the reduction of the pharynx excreted materials can no longer escape by it and thus accumulate within the enteron as they do within the main stem of the protonephridium when it has been cut off from the exterior. The resorption of the anterior organs may be much more extensive than is indicated in Fig. 5. Frequently all of the cephalic structures have been resorbed.

The Turbellaria are usually considered to represent a phase in the phylogeny of the Annelida. That we should see extensive dedifferentiation associated with egg-formation in *Stenostomum* has some significance when the phenomenon known as epitoky in the annelids is considered; for it lends support to the theory that Annelida may have arisen from Turbellaria.

Some experiments were made to test the hypothesis that the reduction of the cephalic organs was necessary in order that material may be supplied to the egg. Specimens were taken that showed incipient ovaries. From these the "heads" were removed. In each case the wound healed, but no new "head" was formed until after the egg was laid. This supports van Cleave's (1929) observation that reconstitution does not occur in female specimens. However, the loss of cephalic tissue that would ordinarily be resorbed did not inhibit the growth of the ovary. In all cases the ovary reached maturity and an egg was laid. The growth of the ovary is therefore not dependent upon the resorption of the cephalic organs. It is suggested that the degree of resorption of cephalic organs, that takes place when the ovary is growing, is correlated with the relative age of the "head" involved. This is a line of observation that one of us plans to follow.

The Appearance of Male Gonads Dependent upon Extrinsic Conditions

Sekera's (1903) suggestion that the appearance of sex is determined by extrinsic factors is supported by our observations. Concerning *Stenostoma leucops*, he says "Aus meinen vieljährigen Beobachtungen, die ich schon vom Jahr 1885 an fort führe, kann ich mir erlauben zu behaupten, dass ein jeder Tümpel nach seiner physischen Beschaffenheit eine bestimmte Zeitdauer hat, in welcher alle Exemplare von *Stenostomum leucops*, *unicolor*, etc. geschlechtlich sich entwickeln. Damit hängt es auch zusammen, dass wir in den nassen Sommerzeiten, wo sich viel Wasser in den Tümpeln anhäuft, mit dem oben erwähnten ungünstigen Umstande für die Ausbildung der Geschlechtsorgane nicht zu rechnen haben" (s. 538).

Specimens have been taken from their natural habitat in May, June, July, August, and September during three years. We have found these to be sexually mature as males only during the last week of August and in September. The species appear to be carried through the winter by zygotes, for extensive collections made in early December and early March yielded no examples of this species.

In contrast to this we have maintained a clone that always presented sexually mature males and at irregular intervals sexually mature females through the year. In addition to this, we have raised sexually mature males from the egg within a period of a month.

It thus appears that certain extrinsic factors of the natural habitat arise in late August which determine the appearance of male gonads, while under laboratory conditions extrinsic factors are present which prompt the development of male gonads throughout the year. *S. œsophagium,* in being sexually mature as males under laboratory conditions, stands sharply in contrast with *S. tenuicauda*. Nuttycombe (1932) has maintained a clone of *S. tenuicauda* for over five years, during which time gonads have not appeared, although specimens collected from their natural habitat were sexually mature in the autumn.

SUMMARY

The male gonad and gonopore lie dorsal to the anterior end of the pharynx. There is no female gonoduct.

The absence of a female gonoduct is correlated with a peculiar mode of copulation.

Egg-formation and egg-deposition bring about a crisis in the life of the individual which is not necessarily fatal. Extensive dedifferentiation and phagocytosis are associated with the development of the egg. After the egg has been laid, a new "head" is developed.

Development of male gonads, in this rhabdocœle, appears to be dependent upon extrinsic rather than intrinsic factors.

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THE RELATION BETWEEN RATE OF LOCOMOTION AND FORM IN AMŒBA PROTEUS[1]

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Mast and Prosser (1932, p. 336) maintain that the rate of locomotion in *Amœba proteus* is highest in monopodal specimens, lowest in multipodal specimens, and intermediate in bipodal specimens, and that ". . . selection of monopodal specimens greatly reduces the variability in rate," but they present no evidence in support of their conclusions. The experimental results presented in the following pages concern these conclusions. They were obtained as follows:

About 50 specimens of *Amœba proteus* were taken at random from a culture, washed[2] three times in redistilled water, and transferred to a Pyrex glass dish containing .001N sodium chloride solution.[3] The dish was then put into a constant temperature apparatus (Pitts, 1932) on the stage of the microscope and left for one hour for adjustment. A single specimen was now selected and projected with a camera lucida on black paper; then the position of the posterior end of the projected image was marked at intervals of one minute, and the outline of the image of the animal sketched at the intervening half minute. This was continued for seven minutes after which it was repeated with each of four other specimens differing in form, and with each of five similar specimens in a number of other dishes. This was continued until records were in hand for 60 specimens, 20 monopodal,[4] 20 irregular monopodal, and 20 multipodal specimens. All of these observations were made on specimens which had been in the sodium chloride solution from one hour to one hour and 45 minutes. The temperature during the observations was 23 ± 0.5°. The results obtained are presented in Tables I and II and Fig. 1.

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[1] This investigation was carried on under the direction of Professor S. O. Mast.

[2] The washing was performed by putting the amœbæ into watch glasses containing redistilled water, allowing them to settle and attach, then removing the water with a pipette, and finally pouring on more redistilled water.

[3] Kahlbaum's purest sodium chloride, accurately weighed and dissolved in water redistilled from a tandem Pyrex glass still (Mast, 1928).

[4] A monopodal amœba is defined as an elongated cylindrical animal, smooth in outline, which moves continually by the projection of a single pseudopod. Marine limax amœbæ of the type used by Pantin (1923) are regularly monopodal in form. *Amœba proteus* readily assumes the monopodal form if it is allowed to become stellate in redistilled water and then transferred to a dilute salt solution (Mast, 1928).

The results in Table I indicate that the rate is highest in monopodal forms and lowest in multipodal forms, and that the variation in rate of the different specimens is least in monopodal forms and greatest in a random sample, the coefficient of variation being 11.4 for the monopodal forms, 17.8 for the irregular monopodal forms, 27.9 for the multipodal forms, and 34.4 for the random sample. Thus it is evident that the variability of any given selected group is considerably less than that of a random sample, and that selection of strictly monopodal forms leads to least variation. The results obtained consequently support the conclusions of Mast and Prosser.

Schwitalla (1924) claims that locomotion in *Amœba proteus* is rhythmical, and he describes a two-fold rhythm—a rhythm of short duration consisting of rapid accelerations and retardations and a rhythm of long duration superimposed upon the short periods. He says (p. 490): "Only rarely will the same rate of locomotion be found to have been sustained for two successive minutes. Sometimes such an uniform rate may be maintained for two or three minutes, and in exceptional cases for five and seven minutes." And (p. 492): "In two successive minutes, a doubled rate, or a rate reduced by one-half occurs with comparative frequency."

TABLE I

Summary of statistics concerning the rate of locomotion of monopodal and multipodal amœbæ.

Form	Number of Specimens	Mean Apparent Rate	Probable Error	Standard Deviation	Coefficient of Variation
		mm. per minute	*per cent of mean*		
Monopodal	20	21.05±.35	1.65	2.30±.25	11.40±1.26
Irregular monopodal	20	19.72±.53	2.69	3.52±.37	17.85±1.96
Multipodal	20	9.44±.40	4.20	2.63±.28	27.86±3.20
Totals and averages	60	15.99±.48	2.99	3.49±.34	34.36±2.35

Hahnert (1932) made a study of locomotion of monopodal specimens of *Amœba proteus* over a period of some twenty minutes and found the rate of locomotion to be surprisingly constant. He found in one specimen, for example, an average rate of 252.6 micra per minute with a standard deviation of only 13.6 micra per minute or 5.4 per cent. Similar results were obtained with nine other specimens tested. He found no indication of rhythmic variation in rate of locomotion like that observed by Schwitalla, and consequently concludes that " the process of ascertaining the rate of locomotion is greatly simplified by using monopodal specimens."

TABLE II

Data Concerning the Rate of Locomotion of Monopodal and Multipodal Amœbæ

Designation of Individuals	1	2	3	4	5	6	7	8	
Monopodal Specimens									
1st minute	20.0	20.5	22.5	22.5	20.0	21.0	23.0	19.5	
2d minute	20.0	18.0	21.0	23.5	21.0	22.0	25.0	21.0	
3d minute	22.0	20.5	21.0	23.0	20.0	21.0	24.0	20.0	
4th minute	21.0	20.5	21.5	22.0	22.0	22.0	23.5	20.0	
5th minute	20.5	20.0	21.5	22.0	22.0	22.0	23.0	19.0	Total average rate
6th minute	20.0	21.0	21.5	20.5	23.0	23.0	22.5	20.0	
7th minute	20.5	20.5		22.5	23.0	23.0	24.5	21.0	
Average rate	20.57	20.14	21.50	22.79	21.43	22.00	23.64	20.71	21.35
Irregular Monopodal Specimens									
1st minute	21.0	10.5	19.5	25.0	24.0	18.5	19.0	23.5	
2d minute	20.0	19.5	18.5	25.0	19.5	15.0	19.0	18.0	
3d minute	16.5	15.0	19.0	18.5	21.5	18.0	16.5	22.0	
4th minute	10.5	9.0	14.5	21.0	21.5	15.0	18.5	23.0	
5th minute	12.5	18.5	16.0	23.5	24.0	16.5	16.0	22.5	
6th minute	18.0	27.5	17.5	25.5	20.0	21.5	21.0	19.0	
7th minute	20.0	12.0	18.0			23.5	22.0	20.0	
Average rate	16.93	16.00	17.57	23.08	21.75	18.29	18.86	21.14	19.20
Multipodal Specimens									
1st minute	12.0	10.0	11.0	6.5	6.5	10.0	9.5	10.5	
2d minute	14.0	11.5	14.0	10.5	7.5	9.0	9.0	16.0	
3d minute	11.0	10.5	7.5	7.0	8.5	13.0	10.5	4.0	
4th minute	8.5	9.0	7.5	8.0	8.5	13.0	16.0	10.5	
5th minute	6.5	5.0	10.0	9.5	8.5	9.5	17.5	11.5	
6th minute	3.5	5.5	9.0	9.5	7.0	8.0	16.5	14.5	
7th minute	3.0	8.0	14.0	7.5	4.0	10.0	17.5	12.0	
Average rate	8.36	8.50	10.43	8.36	7.21	10.36	13.79	11.21	9.77

By examining the rate of locomotion obtained during each of the seven-minute periods of observation of the individual specimens studied in this investigation, some insight is obtained as to the nature and reason for the rhythmical variation in rate which Schwitalla observed and which

Hahnert maintains is not evident in monopodal specimens. By referring to Fig. 3 of Schwitalla's paper it will be seen that there was great variation in the form of the amœbæ used, *i.e.* that some were nearly monopodal, some bipodal, and others multipodal.

It is evident from Table II of the present paper, in which the rates for successive minutes for eight specimens were taken at random from each of the three groups of forms studied, that the variation from minute to minute is much less for monopodal than for irregular monopodal or multipodal specimens. For instance, by comparing specimen 1 of each of the three forms (Table II), it will be seen that the maximum variation within the seven-minute period for the monopodal specimen is 2 mm., or 9.7 per cent of the mean; for the irregular monopodal specimen 10.5 mm., or 62 per cent of the mean; and for the multipodal specimen 11.0 mm., or 131.6 per cent of the mean. This shows that the

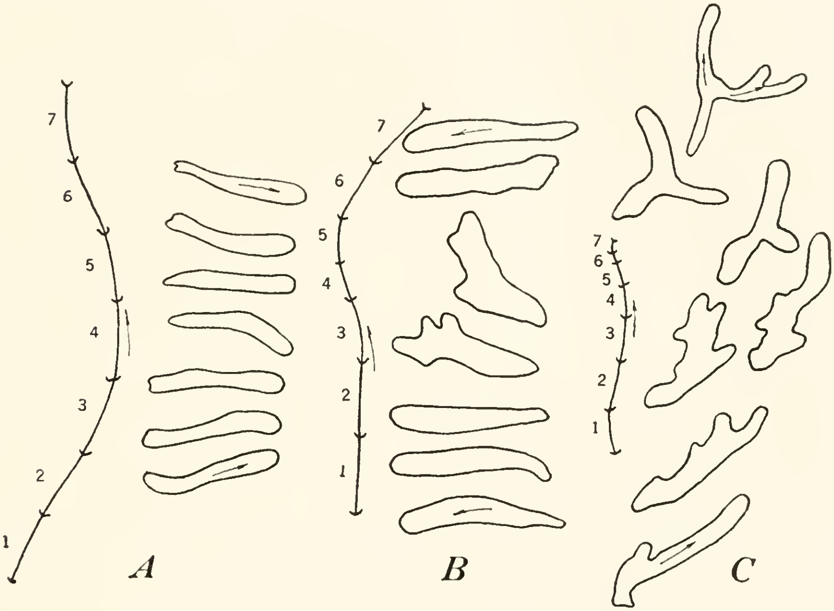

Fig. 1. Camera sketches illustrating the relation between form and rate of locomotion in *Amœba proteus;* curves, projected paths made by these specimens; arrows, direction of locomotion; cross lines on curves, position of the posterior end at minute intervals; outlines, form of the specimens at minute intervals during the time the paths were made.

Note the constant rate of locomotion attending the constant monopodal form (*A*); the decrease in rate as the form becomes irregular (*B*), fourth minute); the increase in rate as the monopodal form is assumed (*B*, seventh minute); and the decrease in rate as irregularity in form increases (*C*).

greater the irregularity of the form of the amœba, the greater the irregularity of locomotion.

Figure 1 gives the records obtained concerning the rate of locomotion of these three specimens, and likewise the outline sketch of them at the intervening half minute. Figure 1*A* indicates clearly that as long as the amœba retains a strictly monopodal form it moves with a fairly uniform velocity. Figures 1*B* and 1*C* demonstrate that in variable specimens of irregular monopodal and multipodal form the marked increases in rate are for the most part due to the assumption of a monopodal or semimonopodal form (Fig. 1*B*, seventh minute); and that the more irregular the form of the animal the slower the rate (Fig. 1*B*, fourth minute, and Fig. 1*C*). It consequently seems probable that the long rhythms which Schwitalla observed were due to change in form of the amœbæ. The short rhythms which he observed were probably due to periodic breaks in the plasmagel sheet, for with each break of this sheet (Mast, 1926) there is a forward spurt of locomotion (Mast and Prosser, 1932). It has, however, been demonstrated that selection of monopodal specimens reduces the variation between rates taken at different times on a single individual. Consequently, in experiments dealing with the effect of environmental factors on rate of amœboid movement, monopodal specimens should be used.

SUMMARY

The rate of locomotion in *Amœba proteus* is highest in monopodal and lowest in multipodal specimens. The average rates obtained for 20 specimens in .001N sodium chloride was 278 micra per minute for monopodal forms, 260 micra per minute for irregular monopodal forms, 125 micra per minute for multipodal forms, and 211 micra per minute for an average of all forms.

The variation in rate is least for monopodal amœbæ and greatest for multipodal amœbæ.

The rate of locomotion of any single individual over a seven-minute period is least variable for monopodal specimens and most variable for multipodal specimens.

The rhythmicity of locomotion in *Amœba proteus* observed by Schwitalla (1924) is largely due to change in form, the rate increasing as the amœba becomes more nearly monopodal and decreasing as it becomes more irregular in form.

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ORDERS OF INSECTS WITH HEART-BEAT REVERSAL

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Introduction

Intermittent (periodic) reversal of direction of heart-beat, formerly regarded as limited to the Ascidea, has been described by Bethe (1927) as normally occurring in *Phoronis psammophora*.

A similar but transitory intermittent reversal in the embryo before normal direction is established has been observed by Bremer (1931–32) in the chick and by Yokoyama (1929) in the silkworm. In the former it appears to be of rare occurrence; in the latter a characteristic of the embryo anticipating the normal periodic reversal to be seen in the prepupa, pupa, and imago.

In response to experimental conditions, reversal of heart-beat during early development of a teleost (*Macropodus viridi-auratus*) was noted by Gowanloch (1923) and in the chick embryo by Johnstone (1925).

Since the publication of my paper (Gerould, 1929)[1] describing the wide-spread and probably universal occurrence of this phenomenon in Lepidoptera after the larval stage with its forward beating has passed, I have observed it in other orders of holometabolous insects. In none of the orders with complete metamorphosis, except possibly Neuroptera (*Corydalis*) not yet thoroughly examined, have I failed to find examples of it in the imago.

Diptera

In a crane fly, *Pachyrhina ferruginea*, peristalsis of the dorsal vessel was observed with extraordinary clearness through the translucent integument of the abdomen. Previous experience with moths had shown that removal of head and appendages does not prevent or seriously affect periodic reversal. The head, wings, and legs were accordingly removed, and peristalsis was observed in abdominal segments 3–5.

I have rarely seen in any insect such a regular series of short alternating phases. In this respect this crane fly resembled the adult

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[1] Attention should be called to an error in this paper on p. 426, line 8, referring to pulse rate; "forward" should read "backward." The sentence should read: "It (average pulse rate in forward beating) is roughly twice as fast as in backward beating."

Bombyx mori rather than the pupa or prepupa of that moth. As shown by Table I taken from my first observation (August 16, 1930; temperature 24° C.), the forward phases averaged about 60 beats, the backward about 36; numbers remarkably similar to averages obtained with young silk-moths, which were 51.7 beats forward, 26.5 backward.

The rate of forward beating in the moth was 10 beats in 17 seconds, backward 10 in 29 seconds, but in the crane fly these data show a nearly equal rate in each direction (average 9.9 seconds forward, 9.5 seconds backward). (See Table II). Of especial interest was the regularity with which the rate of backward beating was accelerated during each backward phase, in contrast to the gradual retardation that took place during the forward phases. Such acceleration was observed in *Bombyx* only during the long backward phase immediately preceding and during pupation.

Table I

Heart-beats of the crane fly, Pachyrhina ferruginea (male)

No. Beats in a Phase		No. Beats in a Phase	
Forward Phase	Backward Phase	Forward Phase	Backward Phase
30 + 14 = 44		63	
	39		35
60 + 16 = 76		57	
	38		38
53 + 16 = 69		63	
	38	56 + 6 = 62	
64			36
	37	51 + 5 = 56	
59			36
	34	65	
51			34
Av. 60.5	37.2	Av. 60.1	35.8

The regularity in the number of beats in the forward phases in this crane fly was soon interrupted by spontaneous activity of the genitalia, which partially inhibited forward beating and reduced the length of the forward phases from an average of 60 beats to about 30, while the alternate backward phases remained of about the same length as previously.

The same preparation 18 hours later (kept in a moist chamber overnight) showed persistent backward beating, broken at first by pauses into groups of about 36 beats. Over 500 beats were counted, and 37 groups of 10 beats were recorded which showed practically no change in rate (10 beats in 10 seconds).

Observations on a drone fly, *Eristalis*, in which beating is extremely rapid, gave among others the following result. Gradual retardation of pulse rate during backward beating is evident. (See Table III).

COLEOPTERA

In the Coleoptera, no published statement in regard to periodic reversal has come to my attention, though Lasch (1913) in studying the larva of the stag-beetle, *Lucanus cervus*, observed in a few normal larvae a single backward wave extending through a few segments, and in one individual injured by artificial stimulation he saw a succession of backward waves extending through several segments, or merely from one into the next one behind it. His observations, however, were apparently limited to the larva, in which normal periodic reversal is not to be expected.

TABLE II

Heart-beats of the crane fly (male), Pachyrhina ferruginea. Temperature 22.5° C.

No. Seconds Required for 10 Beats		No. Seconds Required for 10 Beats	
Forward Phase	Backward Phase	Forward Phase	Backward Phase
Retardation	Acceleration	Retardation	Acceleration
	10.5 8		12 8.5
7 9 10		7 9 9.5	
	11 7		12
7 9 9.5 10		8 10 11.5 10.5	
	9.5 9		9.5 8
7.5 9.5 10.5		9.5 9	
	9		11 7
8 8.5 10.5			
	12 9		

In August 1931 and again in 1932 I made observations on a large beetle, *Prionus laticollis.* The first individual studied was a ♀ found moribund with leg reflexes still feeble; but, as is generally the case in insects, heart action continued long after leg reflexes had ceased.

It was first observed (4:30 P.M.) by slitting each side of the back and pinning back the body wall so that the ventral surface of the dorsal vessel could be seen; there was vigorous double-action or central beating from the front part of the metathorax, forward through the aorta to the head and backward in the abdominal vessel, in which the rates counted successively were 10 beats in 17, 19, 19, 18, 19, 21, 21, 24 seconds. At 5 P.M. there was again central beating. Counts of the forwardly directed waves in the thorax gave 10 beats in 15, 15, 15 seconds, and during the same phase but backward through the abdomen 10 in 18 seconds (temperature 29° C.).

Kept 18 hours in a moist chamber, the head end of the aorta had become dry at 10:50 A.M., but backward peristalsis from the anterior part of the metathorax through the abdomen was proceeding at the rate of 10 beats in 21.5, 22 seconds; for a few minutes double or triple beats went backward from the metathorax, but at 11:13 A.M. the groups of two or three had been reduced to single beats with the rate of 10 in 15, 16, 16 seconds. (Temperature 25.5° C.).

TABLE III

Heart-beats of the drone fly, Eristalis, with abdomen laid open.
Temperature 25° C.

No. Seconds for 10 Beats	No. Seconds for 10 Beats
Forward Phase	Backward Phase
3.5, 3.5, 3, 2.5, 2, 3 →	
	↘ 4, 4.8, 4.5, 5.5, 5, 5, 6, 5, 5.5, 5, 6, 6.5, 5.8, 6, 6.5, 10, 12.5, 9.5
↘ 4, 4, 5.5, 5	←

In August of the following year, a much longer series of observations was made on a ♂ *Prionus laticollis* which was etherized slightly before the removal of mandibles, antennae, legs, and wings (2:33 P.M., August 6; temperature 29° C.). It was first viewed through the translucent anterior segments of the abdomen. Forward beating at the rate of 10 beats in 9.8, 10.8 seconds was followed by backward, 10 beats in 9, 9 seconds. Then a forward phase was followed by a backward phase of over 300 beats beginning at a rate of 10 beats in 6, 7.25, 7.25 seconds.

The backward beating was slightly more rapid than the forward. After amputation of the head, a series of forward waves began at the rate of 10 beats in 8.6 seconds and ended with 10 in 9.5 and 10 seconds. Air sucked into the dorsal vessel interfered with observations, so a preparation was made of the excised back, showing the ventral surface of the abdominal dorsal vessel, the front of which was inflated with air. Thereupon backward beating started in the inflated anterior part and the following records of the duration of 10 beats were taken: 10 in 9.5, 10, 9, 10.5, 9.5, 10.5, 11, 10.5, 11, 11, 11, 12.25, 12, 12.5, 12, 12.4, 13, 13.5, 14, 15, 14, 14.6, 15.6, 16.5, 16.5, 17, 19 seconds. Beating both ways from the middle of the abdomen followed, freeing the front of the tube of the contained air, the rate of beating steadily falling: 10 in 19, 19.5, 20, 20, 21, 21, 23.5 seconds. The preparation, now drying, was moistened with normal saline solution, and completely forward beating began at a more rapid rate, viz., 10 in 5.5, 7, 8, 9, 9, 8.6, 9.5, 9.5, 9.6, 9 seconds. Then completely backward beating through the whole abdomen ensued; 10 beats in 9.5, 9.5, 9.6, 9.5, 9.5, 9.5, 9.5, 9.6, 9.5, 10, 10, 10, 10.5, 9.6, 10.6, 11, 11.4, 11, 11, 11.25, 11.2, 11, 11, 11, 11, 11.5, 11.5, 11.5, 11.5, 11.5, 12, 12, 12.5, 11.8, 12.5, 12.2, 11.8, 12.2, 12.5, 11.5, 12.2, 11.8 seconds. Then central beating; 10 in 12 (double strokes), 11.5, 11.5, 11, 11, 12 seconds (pausing after the seventh). Then forward altogether: 10 in 12, 12, 11, 12, 11, 11, 11.2, 11.2, 10.5, 11.8, 12, 12, 12, 11.8, 11.2, 11.5, 11.4, 11.2, 11.2, 11.2, 11.6, 12, 12, 11.5, 11.8, 12, 12.75, 11.5, 12.5, (moistened) 11, 11, 13, 12, 12.5, 12, 12.75, 12.2, 12.6, 13, 12.6, 14.6, 16.2, 16.6, 17 seconds. On flooding with saline solution, forward beating continued at increased rate: 10 beats in 7.5, 6.5, 6.5, 7.5, 6.5, 7.2, 7, 7, 8, 8, 8.8, 8.2, 9, 9.5, 9.5, 10, 11, 12, 11.5 + (pause after 7). Reverses, a few beats backward, then forward; pause of 10 seconds, then backward; pause of 17 seconds, then forward, then backward. Length of the observations 2.5 hours (2:33–5:03 P.M.), temperature at the end 29° C. as at the beginning.

The following morning the preparation was alive (in moist air) and a long phase of forward beating observed, the rate of which showed gradual and regular retardation. At first there were pauses between each group of about 10 beats. A succession of counts showed 10 beats (forward) in 5.8, 5.8, 6.5, 6.2, 7.4, 6.6, 6.5, 7.5, 6.8, 6.8, 7, 7.5, 7.5, 7.6, 7.6, 8, 8.5, 7.5, 8, 8, 8, 8.2, 8, 9, 10, 10.5, 11, 10, 10, 10 seconds. There were now less than 10 beats in a phase, and soon groups of 2 beats came; counts of 10 pairs (forward) in 17.5, 19.5, 18, 19, 20, 20, 20, 20, 21, 20.6, 21, 22 seconds. Now single beats replaced pairs and counts of 10 gave 24, 25.4, 24.5, 28, 28, 25.5, 25, 27, 29, 28, 29, 26.5, 29, 33 seconds and beating stopped. Saline solution was applied, and a series of converging beats conflicting at the third abdominal segment (backward in front of the third segment but still forward behind it) gave the following series of counts: 10 beats in 9.5, 8, 7.5, 8, 7, 8, 7.5, 7.5 seconds.

The same preparation 24 hours after the first observations, temperature 28.5° C., showed a long run of short groups of backward beats separated by pauses of about 10 seconds. There were groups of 7, then of 5 in 3, 4, 4, 3.8, 3.2, 3, 2.5 seconds.

Table IV

Heart-beats of solitary wasp, *Sphex*, without head or wings, first three abdominal segments observed. Temperature 28° C. First phase, observed in forward beating, lasted 25 minutes.

Forward Phase	Backward Phase
Rate 10 beats in 2 seconds	10 beats in: 2.6, 2.5, 2, 2.5, 2.5, 2, 2.5, 2.2, 2.6, 2.5, 3, 4, 3, 3.5, 2.6, 2.6, (lags), 5, 4.5, 5, 8, 9 (in flashes of 2), 14, 15, 17, 16, 26, 27.8, 32, 44.5, 83, 82 seconds, length of the phase 32 minutes, last half in groups of 2 with longer and longer pauses between successive pairs.
10 in 11 seconds 11 seconds 11 seconds	

Hymenoptera

Observations on individuals of this order are less easy and satisfactory than in other groups of higher insects for two reasons, the great length of the phases and rapidity of pulsation. Ants and bees, with their short dorsal vessels with extremely rapid beating, are particularly unfavorable. I have not yet been able to get reliable data on reversal in either group.

In a large solitary wasp, *Sphex*, however, with translucent tergum at the base of the abdomen, satisfactory proof of reversal was obtained, as Table IV shows.

This shows a backward phase of 32 minutes of exceedingly rapid beating, gradually diminishing in rate until at the end of the phase it became very slow.

An ichneumon fly, *Opheltes glaucopterus*, found moribund, reaction of appendages having ceased, gave some excellent data showing unmistakable periodic reversal, with the rapid beating and long phases characteristic of this order. The heart was observed through the translucent tergites; body walls, head, and appendages were intact. The first observation was of a long forward phase of 27 minutes (11:23 A.M.–12 M., temperature 26.5° C.). Due to the rapid rate, groups of 20, rather than 10 beats were measured; 20 in 15 seconds at first, in 16 seconds at the end (12 readings). A second observation began at 3:20 P.M. with a backward phase of 47 minutes during which the duration of groups of 20 beats remained nearly constant, with very slight acceleration (average of 20 beats in 22.2 seconds for the first 5; 18.9 seconds for the last 5 measurements). At 5–5:08 P.M. measurements of the rate of another clearly backward phase gave an average of 18.5 seconds for 20 beats with no change. (Temperature 26.5° C.).

ORDERS PROBABLY WITHOUT HEART-BEAT REVERSAL

Examples of adult Neuroptera and Odonata have shown no reversal. A female dobson fly, *Corydalis cornuta*, the large size and comparatively slow pulse of which make it favorable for study, was under observation for 40 minutes; no reversal occurred. One of the Chrysopidae translucent enough for external examination showed no heart-beat reversal.

In a dragon fly, *Plathemis trimaculata*, pulsation of the dorsal vessel, observed in three males and one female, was only forward.

SUMMARY

Representatives of four orders of holometabolous insects, Diptera, Coleoptera, Lepidoptera, and Hymenoptera have shown periodic (intermittent) heart-beat reversal in the pupa and imago. In an adult crane fly, the number of forward beats per phase exceeded the backward, but the average rates of beating in each direction were almost equal. Gradual retardation of rate occurred within each single forward phase, slight acceleration within each backward phase. In the adult beetle there was likewise no marked difference between the average rates backward and forward. A very gradual retardation in rate was observed during each phase whether backward or forward. Central beating in both directions from the pulsating vessel of the metathorax or from the middle of the abdomen occurred. A few converging waves conflicting at the third abdominal segment were observed. Saline solution quickened the rate. There was an occasional tendency to beat in groups of two without change of rate. Pauses sometimes broke up a long phase, each being of about the same length as the groups among which they were interpolated. In Hymenoptera, long backward and forward phases, each a half hour or more in length, were characteristic of *Sphex* and *Opheltes*. The rates were rapid. In *Sphex* the rate backward was gradually retarded; in *Opheltes* the rate during any long phase was nearly constant, though during one backward phase of 47 minutes there was slight acceleration.

In general, normal reversal occurs independently of the central nervous system and is essentially myogenic. Stimuli from the head, wings, legs, and genitalia, however, secondarily affect peristalsis in detail, producing variations in rate, length of phase, number of beats in a phase, and occasionally probably reversal. Central beating does not depend upon special ganglia but upon local regions of higher irritability (or greater local inflow of haemolymph from the pericardium), which vary somewhat even in the same individual.

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THE MORPHOLOGY OF THE LATER STAGES OF BALANUS CRENATUS BRUGUIERE

LUDWIG E. HERZ

The development of barnacles has occupied the attention of numerous workers since 1830, when J. V. Thompson discovered that nauplii appeared in their development. This similarity in development with Crustacea led to the transfer of the class Cirripedia from the phylum Mollusca, where it had been classified by Linnaeus, Cuvier, and others, to the phylum Arthropoda. In 1843 Burmeister made an important contribution when he found that in *Lepas* the larvæ passed through two totally different stages, the nauplius and the cypris. He had received what he termed a " ball of *Lepas* " on which the cypris stages had just attached and the adults were dismissing the newly hatched nauplii. He thus missed the intermediate stages in the developmental series. In 1875 Dr. R. von Willemoes-Suhm studied the life history of *Lepas fascicularis* while making a cruise from Japan to Sandwich with the Challenger expedition. Concerning this subject he wrote: " My object is to give an idea of the whole development of one Lepas as accurately as possible which seems never to have been done as yet as our knowledge consists of fragments collected mostly in the same way in which Burmeister gained his information." Dr. von Willemoes-Suhm (1875) found that six different nauplius stages and one cypris stage appeared in the development of *Lepas fascicularis,* a pedunculate barnacle of the suborder Lepadomorpha. His report, owing to his untimely death, was published by a co-worker. No mention was made of the transformation of the cypris stage into the adult form. No records of a complete study of the development of an operculate barnacle in the suborder Balanomorpha have been found as yet, nor has any other study of another species of barnacle approached the degree of completeness attained by von Willemoes-Suhm.

This investigation takes up the life history of *Balanus crenatus* Bruguiere of the suborder Balanomorpha, from the newly-hatched nauplius to the adult form. Eight nauplius stages and one cypris stage were found. This study was undertaken upon the suggestion of Dr. Harold Heath of Hopkins Marine Station, Pacific Grove, California, and carried on at Stanford University during the academic years of 1928-29 and

1929–30, and at Hopkins Marine Station during the summer of 1931.[1]

Reasons for the success of this research and the failures of earlier workers may be briefly told. Along the shores of the ocean where many species of barnacle abound and reproduce, it is exceedingly difficult to segregate the nauplii of the different species that are secured by means of plankton hauls since there is a great similarity in their sizes and general structure. The previous investigators therefore attempted to rear in aquaria the newly-hatched nauplii that were taken from the mantle cavity of gravid adult barnacles to secure the complete series of stages in one species, and eliminate the possibility of including the nauplii of other species. This method did not prove successful since the nauplii invariably died after moulting two or three times. The writer met with these same results when efforts were made to isolate and raise the first stages of five of the species of barnacle[2] found on the shores of Monterey Bay. All sorts of containers ranging in size from Syracuse glasses to gallon battery jars in which fresh and balanced or rotted sea water was placed were used as aquaria, but in no case was it possible to secure any moults beyond the third stage. The larvæ would usually remain alive in the third stage for periods of time ranging from eight to twenty-four days. Another method used was to isolate the newly-hatched stages of nauplii in quart jars, cover the jar mouths with cloth, and anchor the jars to floats at various depths, ranging from a few inches to ten feet. The nauplii invariably died in the third stage. These experiments indicated that: (1) the nauplii are very delicate; (2) their mortality rate is very high; (3) their most favorable environment is limited to the open ocean near the shoreline where the water is well aërated by the dashing of the breakers over the rocky shore. Success was attained, however, when experiments were transferred to the hardier specimens of the two species of barnacle that are found in the sloughs of San Francisco Bay. These species are *Balanus crenatus* Bruguiere,[3] and *Balanus glandula* Darwin. *Balanus crenatus* was selected for investigation, as its breeding period extended from March until November, while *Balanus glandula* discontinued breeding after June. Ripe eggs were taken from the mantle cavities of these barnacles and placed in aquaria. After the passage of three days, nauplii in the fourth or fifth stages were found. The later stages were found after the lapse of seven to ten days, and the newly-attached adult form barnacles appeared after thirteen or fourteen days. The times that are required for the development of the later stages of *Balanus crenatus* no doubt vary with the temperature. The times recorded in this paper were secured by observing aquaria specimens that were hatched from ripe eggs. One-gallon battery jars were used as aquaria. These were kept near the windows of a laboratory located on the second floor of the Zoölogy building at Stanford University. A number of thermometer readings taken during July and August, 1930, showed that the temperature of the aquaria did not vary greatly from day to day. The lowest temperature recorded during this time was 17° C., while the highest was 21°. This fluctuation in temperature is perhaps less than that of the slough waters, which are often warmed considerably when the incoming tides flow over exposed and warmed mud flats.

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[1] The writer wishes to thank Dr. Heath for his direction throughout the work, and wishes to express his appreciation to the following: to Dr. H. A. Pilsbry of the Philadelphia Academy of Natural Sciences for identifying specimens of barnacle; to Professor G. F. Ferris of Stanford University for valued advice; to Dr. W. K. Fisher for the use of the facilities of Hopkins Marine Station; to Dr. Tage Skogsberg for valued advice on drafting the plates of this research.

[2] The five species were *Mitella polymera* Sowerby, *Balanus glandula* Darwin, *Balanus tintinnabulum* Linnæus, *Tetraclita squamosa rubescens* Darwin, and *Lepas Hillii* Leach.

[3] The type locality of *Balanus crenatus* is the coast of England. It is found in the Arctic Ocean; North Atlantic south to Long Island Sound; Bering Sea and North Pacific south to Santa Barbara, California, and Northern Japan.

The specimens were easily located, since the nauplii are positively phototropic and the cypris attach on the illuminated side of the aquaria.[4] The nauplii were removed with a pipette to a slide and mounted in glycerine. The cypris stages were mounted in euparal and balsam. Bouin's solution was used as fixing medium. Alum carmine and Delafield's hæmatoxylin were used as stains for the whole mounts of the cypris stage.

The eggs are found in gelatinous masses in the branchial cavity. These are oval, measuring .19 mm. in length and .12 mm. in width. The freshly-spawned eggs are light gray in color. As the eggs ripen, the masses assume a definite shape, and the eyespots and appendages are easily distinguished. The egg membranes are ruptured by the movements of the appendages of the nauplii.

The first stage nauplius measures .22 mm. in length from the anterior portion of the carapace to the tip of the thoraco-abdominal process. It measures .12 mm. in width across the carapace immediately posterior to the frontal horns. An eyespot appears in the mid-section of the anterior portion of the nauplius. It is square in outline, and is situated in the center of a bi-lobed structure. The oral lip is situated immediately posterior to the eyespot. This assists in injestion. The thoraco-abdominal process is quite rudimentary. It projects but slightly beyond the posterior tip of the carapace and ends as a two-pointed structure. The alimentary canal is a simple appearing tubular organ.

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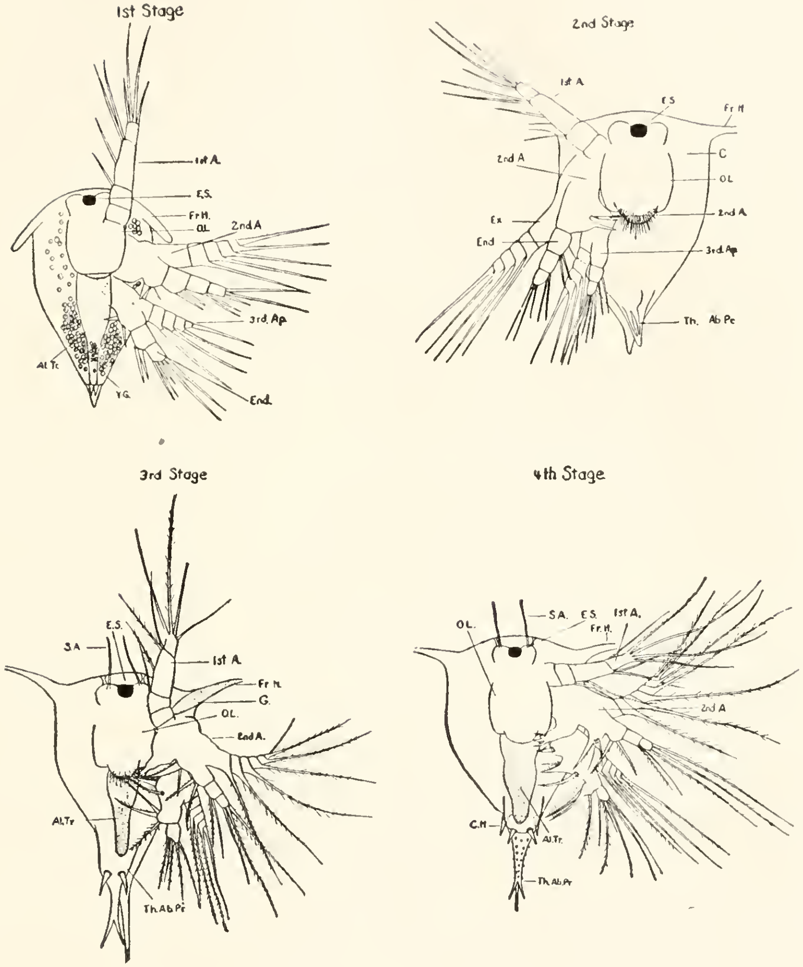
[4] This result differs from the findings of Dr. Paul J. Visscher, who reports that the cypris of *Chthamalus fragilis*, *Balanus improvisus*, and *Balanus amphitrite* are negatively phototropic at the time of attachment.

PLATE I


1st Stage
1st A.
E.S.
Fr.H.
OL
2nd A
3rd. Ap.
Al. Tr.
Y.G.
End.
2nd Stage
1st A
E.S
Fr H
2nd A
C
OL
2nd A
Ex
End
3rd Ap.
Th. Ab Pr.
3rd Stage
E.S.
S.A
1st A.
Fr H.
G.
OL.
2nd A.
Al.Tr.
Th.Ab.Pr.
4th Stage
OL.
S.A.
E.S.
1st A.
Fr.H.
2nd A
C.H.
Al.Tr.
Th.Ab.Pr.

1st A., First Antenna
2nd A., Second Antenna
Ex., Exopodite
End., Endopodite
E. S., Eye Spot
Fr. H., Frontal Horn
C., Carapace

O. L., Oral Lip
3rd Ap., Third Appendage
Th. Ab. Pr., Thoraco-Abdominal Process
Al. Tr., Alimentary Tract
G., Gland
Y. G., Yolk Globule
S. A., Sensory Appendage

C. H., Caudal Horn

The mouth is located at the oral lip and the anus between the two-pointed extremities of the thoraco-abdominal process. The nauplius is propelled by means of three pairs of appendages. The anterior pair is uniramous and the second and third are biramous.

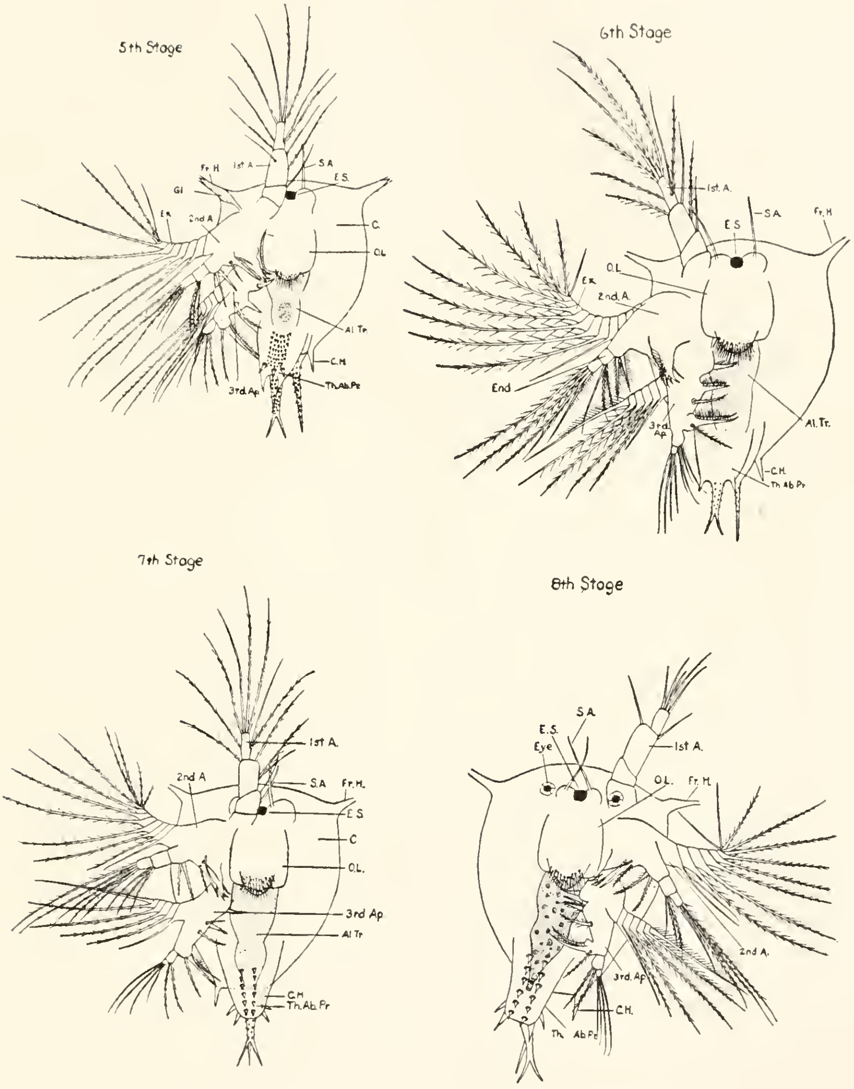
The second stage is slightly larger than the first. It measures .20 mm. in length and .14 mm. in width. The thoraco-abdominal process is larger and more clearly visible. The anterior horns, which pointed in a posterior direction in the first stage, project outward at right angles to the anterior-posterior line. The appendages have no new structures.

The third stage measures .31 mm. from the anterior end of the carapace to the tip of the thoraco-abdominal process. The width of the carapace immediately posterior to the anterior horns is .15 mm. The thoraco-abdominal process has elongated greatly. It is made up of two distinct parts, a longer single-pointed dorsal structure and a forked ventral process. Two stout spines project from the mid-section of the ventral process. The first appendage has no new structures. Setæ have developed on three of its spines. Two new spines are seen on the exopodite of the second appendage, making a total of seven on this ramus. A stout three-pointed, spine-bearing structure appears on the gnathobase. This plays an important part in injestion of food. One new short spine has developed on the exopodite of the third appendage, making a total of five on the ramus; a new spine appears on the second segment of the endopodite of this appendage.

Two large spines have developed at the posterior end of the carapace of the fourth stage nauplius. This stage measures .27 mm. in length from the anterior tip of the carapace to the base of the caudal horns. The anterior horns do not stand out as prominently as in the previous stage. New spines appear on the following: on the proximal portion of the outer side of the distal segment of the first appendage; on the basal segment of the endopodite of the second appendage; a minute spine, which is not visible on all specimens, on the distal segment of the endopodite of the third appendage, making a total of six on this segment.

The length of the fifth stage from the anterior tip of the carapace to the base of the caudal spine is .27 mm. The width posterior to the anterior horns is .22 mm. Three new spines appear on the ventral side of the forked structure of the thoraco-abdominal process anterior to the two that appeared in the fourth stage. The two distal segments of the first appendage have undergone considerable modification. The fourth segment is much narrower than the third. It now bears seven spines. Four spines project from the third segment, the proximal spine of the outer two being not visible on most specimens. New spines appear on both rami of the second appendage. Nine spines, two of which are

PLATE II


5th Stage
Fr. H
1st A
S A
E.S.
Gl
Ex
2nd A
C
O.L
Al. Tr.
C.H
3rd Ap
Th.Ab.Pr
6th Stage
1st. A.
S A
Fr. H
E S
O.L.
Ex
2nd. A.
End
3rd. Ap.
Al. Tr.
C.H.
Th Ab Pr
7th Stage
1st A.
2nd A
S.A
Fr. H.
E S
C
O.L.
3rd Ap
Al Tr
C.H
Th.Ab.Pr
8th Stage
S.A.
E.S.
Eye
1st A.
O.L.
Fr. H.
2nd A.
3rd. Ap
C.H.
Th. Ab Pr

1st A., First Antenna
Fr. H., Frontal Horn
GL., Gland
Ex., Exopodite
2nd A., Second Antenna
End., Endopodite
3rd Ap., Third Appendage

S. A., Sensory Appendage
E. S., Eye Spot
C., Carapace
O. L., Oral Lip
Al. Tr., Alimentary Tract
C. H., Caudal Horn
Th. Ab. Pr., Thoraco-Abdominal Process

new, project from the exopodite. Two new spines have developed on the distal segment, and one new spine on the third segment of the endopodite. No new spines appear on the rami of the more conservative third appendage.

The sixth stage nauplius measures .35 mm. in length from the anterior tip of the carapace to the base of the caudal horns and .34 mm. in width. The first appendage has changed little. The exopodite of the second appendage bears eleven spines, an increase of two over the previous stage. One new spine projects from the exopodite of the third appendage, making a total of six on this ramus. Two new spines project from the thoraco-abdominal process external to the three that appeared in the last stage.

The seventh stage measures .46 mm. in length from the anterior tip of the carapace to the base of the caudal horns and .35 mm. in width.

TABLE I

Tabulation of number of spines on first appendage and on exopodites of second and third appendages.

Stage	First Appendage	Ex. Second Appendage	Ex. Third Appendage
1	8	5	4
2	8	5	4
3	8	7	5
4	9	7	5
5	12	9	5
6	11	11	6
7	12	12	6
8	?	12	6

The most important of the new structures apparent in this stage is the anlage of the six pairs of swimming appendages which function in the cypris stage. Six pairs of stout spines have developed on the ventral surface of the thoraco-abdominal process, giving the anlage added protection. The second appendage is the only one that has a new spine. This projects from the exopodite, making a total of twelve on this ramus.

The eighth stage measures .50 mm. in length, and .41 mm. in width. Two eyes have developed lateral to the median eyespot. The undifferentiated anlage which appeared in the thoraco-abdominal process of the previous stage has metamorphosed into rudimentary swimming appendages. These are found within the walls of the thoraco-abdominal process. The three nauplius appendages have, with the exception of the first, undergone no change. The spines of the first appendage have degenerated and become short and stubby.

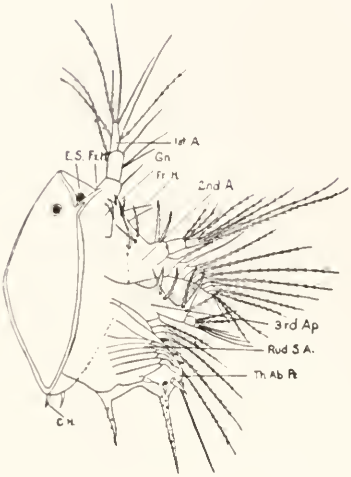
A tabulation of the number of spines on the first appendage and on the exopodites of the second and third appendages in Table I shows the totals given. An interpretation of the data listed indicates that the number of spines on the appendages of each stage is quite definite and the rate of increase during the development is orderly. The number of spines listed for the first appendage of the fifth stage is open to question, since a minute spine which is not present on all specimens of this stage is included in this total.

The cypris stage measures .55 mm. in length and .24 mm. in width. Its valves have been formed through a folding of the carapace of the nauplius stage. The first appendage has become modified and now is used as an adhesive organ. The two biramous swimming appendages of the nauplius stages have disappeared. Their rudiments appear to be formed into the mandibles and maxillas in the metamorphosis into the adult form barnacle. Two long slender organs extend ventralwards from the region of the eyes. These are .13 mm. in length and are the sensory appendages of the nauplius stages. These are moved back and forth as the cypris moves along and appear to act as feelers. Four eyespots appear in the forward end of this stage. Two of these are located along the median line of the body on the walls of the valves. These quiver constantly during the movements of the cypris. Two smaller eyespots are present dorsal to these two. The larger eyespots and the first antennæ are attached to the valves by some eight or ten muscular strands. The abdomen, bearing the six pairs of propelling appendages, is a very flexible organ. It moves on a pivot formed by a set of muscles which attach the abdomen to the valves near the mid-ventral portion of the body. The abdomen is capable of being completely withdrawn within the protective walls of the valves during quiescent periods; in times of movement the abdomen turns ventrally, placing the appendages in position for vigorous movement, which causes the cypris to dart ahead at a rapid rate. The abdomen is prevented from turning outward too far by a stout muscle (R. M. of ninth stage on Plate III) that connects the abdomen with the dorsal walls of the valves near the anterior end of the cypris. An enlarged area of the enteric tract that may be termed the stomach appears just posterior to the insertion of the dorsal retractor muscular strand. The enteric tract passes posteriorly and the anus appears between the sixth swimming and the seventh rudimentary appendages. A pair of shell glands are located anteriorly to the adductor muscles. A small duct passes dorsally from the gland. Its subsequent course was not definitely determined.

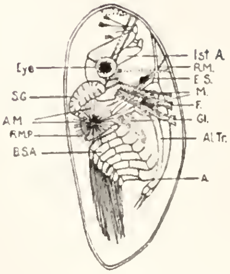
The cypris stage, when on the point of transforming into the adult form, settles on some hard surface. The first antennæ are extended

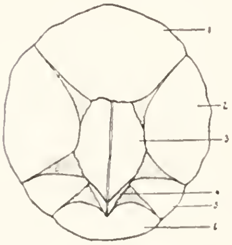
PLATE III

8th Stage
Side View

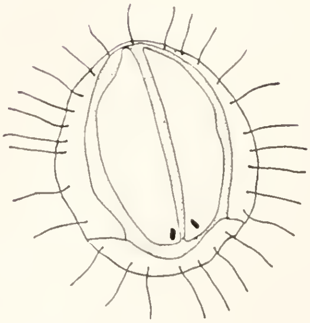

1st A
E.S. Fr.H
Gn
Fr. H
2nd A
3rd Ap
Rud S A.
Th Ab Pr
C H

9th Stage


Eye
SG
A M
RMP
BSA
1st A
R.M.
E S.
M.
F.
Gl.
Al Tr.
A



Adult Barnacle



Newly Attached Barnacle

E. S., Eye Spot
C. H., Caudal Horn
Fr. H., Frontal Horn
1st A., First Antenna
Gn., Gnathobase
2nd A., Second Antenna
3rd Ap., Third Appendage
Rud. S. A., Rudimentary Swimming Appendages
Th. Ab. Pr., Thoraco-Abdominal Process
S. G., Shell Gland

A. M., Adductor Muscle
R. M. P., Rudimentary Mouth Parts
B. S. A., Biramous Swimming Appendages
Ist A., First Antenna
R. M., Retractor Muscle
E. S., Eye Spot
M., Muscle
F., Food
Gl., Gland
Al. Tr., Alimentary Tract

Anus, Anus

far out from the valves and the two pairs of eyespots are found near the ventral portion of the body. The muscles and ligaments that attach these parts to the valves are extended to their fullest extent. These are eventually ruptured and the antennæ and the larger of the two pairs of eyespots are cast off. The remaining portions of the cypris are loosened from the valves by the kicking action of the appendages, and these parts slip out from the old chitinous layer that surrounds them. A gelatinous substance exudes from the body membrane at the base or point of attachment. This develops into the compartments. The number of the compartments is not clearly visible when they are first layed down, but they become more distinct as the barnacle grows. The plates merge at the base and form a thin calcareous layer which lines the base of the barnacle. The scuta and terga develop immediately above the gelatinous matrix of the compartments. In the adult barnacle the scuta and terga are considerably below the tips of the six compartments. This appears to be due to the upward growth of the compartments, rather than to an invagination of the membrane from which the terga and scuta develop. The rapidly-moving swimming appendages of the cypris stage have transformed into slow-moving food-securing organs.

CONCLUSION

Balanus crenatus Bruguiere passes through eight nauplius stages and one cypris stage in its development from the egg to the adult form. The time necessary for this to take place is from two to three weeks.

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